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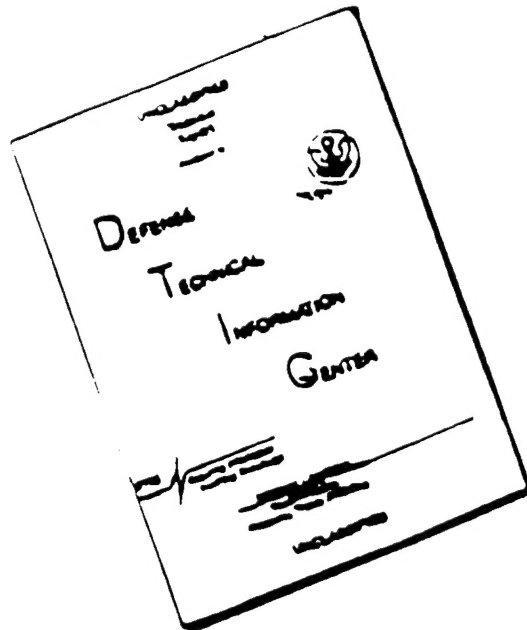
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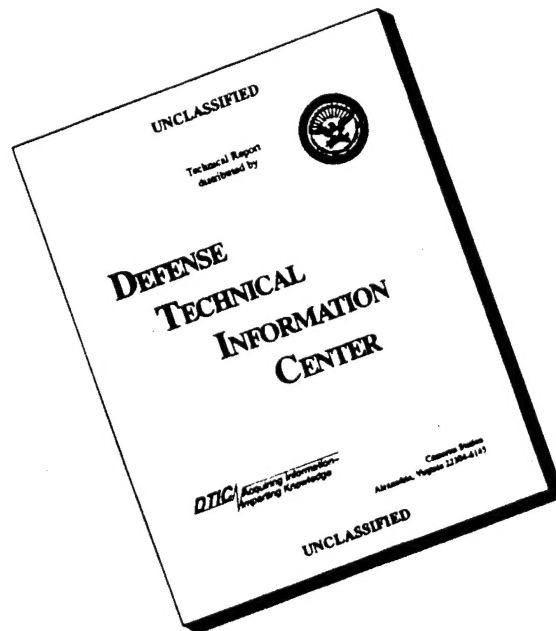
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FOREWORD

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FORWARD

Forward to Final Report

The formal agreement to establish the Belize-United States Epidemiologic Research Center (ERC) was signed on 12 December 1989 by the Honorable Drs. Said Musa, Minister of Foreign Affairs, and Theodore Aranda, Minister of Health and Urban Development, representing the Government of Belize, and The Honorable Robert G. Rich, Jr., Ambassador, representing the Government of the United States. The signing followed approximately one year of meetings and discussions between Dr. Jose Antonio Lopez, who was then serving as the Director of Health Services during Dr. Errol Vanzie's absence on sabbatical at Tulane University, and Dr. Llewellyn J. Legters, Professor and Chairman of Preventive Medicine and Biometrics at the Uniformed Services University of the Health Sciences (USUHS). The ERC was established with funds from a grant to USUHS from the U.S. Army Medical Research and Development Command.

The stated purpose of the ERC was to conduct epidemiological research on infectious diseases of major public importance to Belize. It was to be comprised of a laboratory base, staffed and equipped to address research questions useful in the control of infectious diseases and to conduct field investigations of epidemic disease occurrences. The laboratory, backed-up by U.S. facilities, would augment current public health laboratory and epidemiological capabilities of the Belize Ministry of Health. An integral and significant aspect of the program was provision for education and training of Belizean scientists and technicians in short-term and degree-granting programs in the U.S., and reciprocally, of selected USUHS medical and graduate students during short-term rotations in tropical medicine and field epidemiology in Belize. Besides the technology transfer that would result from the education and training programs, the principal benefit to Belize was anticipated to be the development of new information necessary to refine strategies directed toward prevention of the principal infectious diseases endemic in the country.

Approximately one year after the signing of the agreement, on 29 November 1990, the Central Medical Laboratory (CML), newly-renovated with funds from the grant, was dedicated at a ceremony presided over by The Honorable Doctor Theodore Aranda, Minister of Health and Urban Development, and The Honorable Eugene Scassa, U.S. Ambassador. Dr. Richard Krieg from USUHS was assigned to Belize in January 1991 as Co-Director of the ERC. And so began a four-year period of collaborative research and teaching in tropical infectious diseases of great mutual benefit to both the Belize and U.S. Governments.

This marks the end of project funding by the U.S. Army, but it also marks the continuation of projects under other sponsorship, especially National Aeronautics and Space Administration sponsorship of a project to apply remote-sensing and geographic information system technologies in Belize's National Malaria Control Program.

The program has been extraordinarily successful. Four Belizean physicians, Drs. Peter Craig, Mohan Kishore, Michael Pitts and Jorge Polanco, successfully completed Master of Public Health or Master of Tropical Medicine and Hygiene degrees at USUHS. Nine fourth-year USUHS medical students and four graduate students completed training rotations in tropical medicine in Belize. Transfers of technology accomplished under the cooperative agreement have included initiation of testing for HIV-1 in the Belize Blood bank, a function now performed by the CML. The ERC also initiated testing for hepatitis B surface antigen in the Belize blood bank, screening over 1000 specimens before this procedure was incorporated into the CML. Transfers of technology have also been accomplished in the area of ELISA testing for Rotavirus in stool, hepatitis B surface antigen, anti-hepatitis B core antigen (anti-HBc), anti-hepatitis A (both total and IgM), anti-hepatitis C, and antibody against Trypanosoma cruzi, the agent of Chagas' disease. The technologists and secretary of the ERC have also received significant training in computer programs, including word processing, managing a database, and the production of maps on the computer. The accompanying list of Abstracts of Presentations and Publications speaks to the research productivity of Belizean and U.S. scientists involved with the project.

Many people, both Belizeans and Americans, have contributed in very significant ways to the program's success. Besides those already mentioned, I would be remiss not to mention Mr. Fred Duncan of my staff, who along with Mr. Walwyn Tillett, designed and supervised the renovation of the CML; Drs. Greg Castillo, Medical Chief of Staff, and Filiberto Cawich, Head of Internal Medicine, who gave generously of their time, knowledge and skills in mentoring USUHS students during rotations in Belize; and last, but certainly not least, the dedicated and hard-working ERC staff of Linda Reyes, Shilpa Hakre, Ruth Jaramillo, Rosita Miller, and Ernest Black.

The project in Pakistan has also been a source of immense productivity. Even though there have been many hurdles to overcome including language, cultural and those relating to international politics, work has proceeded on very important projects. With the signing of a memorandum of understanding in 1984 between the Army Medical College and the Uniformed Services University of the Health Sciences, a period of intense research in the areas of causes of febrile illnesses and etiologies of hepatitis was begun. In 1986, construction on a new laboratory was completed and the building was inaugurated. During the period 1987-1989, a number of investigators conducted research projects with their Pakistani counterparts. These included work on etiologies of febrile illnesses among patients admitted to the Combined Military Hospital, etiologies of hepatitis among Pakistani soldiers admitted to Combined Military Hospital, and a number of entomological studies. In addition, two well studied outbreaks of hepatitis were evaluated in the field at Abbottabad and Sargodha. Specimens from these two outbreaks have laid the basis for important work in the area of hepatitis E virus (HEV) during the period 1989-1995.

In collaboration with the National Institutes of Health in Bethesda and the Walter Reed Army Institute of Research in Washington, studies on specimens from Pakistan have resulted in 1) characterization of HEV including complete sequencing of the genome, 2) comparison of the Pakistani strain with other strains from around the world, 3) expression of structural proteins of HEV in baculovirus, 4) development of a sensitive diagnostic test from these expressed proteins, 5) development of a prototype

vaccine against HEV from these expressed proteins, 6) characterization of the excretion of HEV in feces using IEM and also PCR and 7) characterization of the pattern of antibody to HEV in persons infected in these outbreaks.

My hope and expectations are that this is only a milestone on a long and productive road of continued cooperation between our respective institutions.

A handwritten signature in cursive script, appearing to read "Llewellyn J. Legters".

Llewellyn J. Legters, M.D., M.P.H.
Professor and Chairman
Preventive Medicine and Biometrics
Uniformed Services University
of the Health Sciences

ABSTRACT

Between December 1, 1989 and May 31, 1995 a number of research projects were accomplished in Belize. The area that was most thoroughly studied was that of hepatitis. In response to reports of a number of cases of hepatitis around a banana plantation in 1990, a serosurvey of workers and their families was conducted. This revealed that up to 70% of workers had been infected with hepatitis B and almost all had been infected with hepatitis A. Later, the prevalence of hepatitis A, B, and C was investigated among the Belize Defense Force, Hepatitis B among pregnant women, health care workers, and school-aged children. A study of acute cases of jaundice was conducted over one year in the district of Stann Creek where citrus and banana plantations exists. Two hepatitis B vaccine trials were conducted among the Belize Defense Force. Other areas of investigation included malaria, leishmaniasis, diarrhea, and Chagas' Disease. A number of entomologic studies were conducted including the use of remote sensing to predict houses with a high likelihood of malaria vectors. Work begun earlier in Pakistan was continued and resulted in the sequencing of the genome of an isolate of hepatitis E, the subsequent development of serologic assays for hepatitis E, and a PCR for Hepatitis E in feces. These tools have been used to investigate 2 outbreaks of hepatitis E in Pakistan.

INTRODUCTION

The objective of this grant was to establish, operate and manage research and teaching programs in overseas locations where the USUHS has established bilateral research agreements. These centers served as sites wherein research projects of programmatic interest to the USAMRDC in the field of tropical infectious diseases can be conducted by USAMRDC and USUHS personnel in collaboration with host national counterparts. The program also provides the opportunity to transfer technology to the host-country scientists and technicians through short-term and degree-granting programs. It also provided USUHS medical students, Master of Tropical Medicine and Hygiene students and Doctoral Candidates in Medical Parasitology and Vector Biology opportunities to obtain practical experience with tropical infectious diseases of the Western Hemisphere.

In Belize, the primary objective was to establish and maintain an Epidemiological Research Center (ERC) for infectious disease research and teaching in the Ministry of Health, Central Medical Laboratory (CML), Belize City.

Background

Belize is located on the east coast of Central America at the base of the Yucatan Peninsula, bordered on the south and west by Guatemala, on the north by Mexico, and on the east by the Caribbean Sea. The jungle-covered Maya Mountains occupy the southwestern part of the country, rising to 1122 meters at Victoria Peak; the rest of the country is low, crop or scrub-covered coastal plains. Belize was founded as a buccaneer settlement and entreport. Belize is an English-speaking country, which gained independence from Great Britain in 1981. The population of approximately 195,000 people is a mixture of Mayans, Garifuna (Afro-Amerindian), Blacks, East Indians, Creole and Caucasians. About half of the population lives in the largest city, Belize City, on the

Caribbean coast. The capital, Belmopan (population about 5,000) was built inland as a Federal District after a devastating hurricane in 1961 destroyed the former capital, Belize City. Several smaller cities; i.e., Punta Gorda, Dangriga, Hill Bank, Orange Walk, San Ignacio and Indian Church, are scattered through the coastal plain.

Medical care is provided by a socialized medical system, centered around local health clinics and district hospitals in the smaller cities and towns and a large central hospital in Belize City. Emergency cases (mainly surgical) are brought to Belize City Hospital for care by air ambulance, provided to the government by the Christian groups that has established missions throughout the country. The Belize City Hospital was built about 1930 and is a two-story building backed on the sea. It has about 200 beds, of which about 60% are dedicated to acute surgical patients. The hospital is divided into male, female and pediatric wards for both surgery and medicine. There are also neonatal and intensive care wards. A small chemistry, hematology and immunohematology laboratory for acute diagnostic procedures is located in the hospital. The majority of diagnostic and public health laboratory procedures are performed at the Ministry of Health, Central Medical Laboratory (CML) located about 3 miles north of the city. Current laboratory capabilities include: malaria smears (about 29,000/year), bacteriologic cultures, routine biochemistries, and HIV antibody testing using commercially available ELISA kits.

Some of the research objectives were to:

- a) Determine the etiology of acute febrile illnesses ; determine antibody prevalence against various arthropod-borne viruses (e.g., EEE, VEE, WEE, SLE, MAY, dengue, YF, VSV, etc.), and leptospirosis; maintain surveillance for epidemic disease due to arthropod viruses, especially dengue.
- b) Determine the etiology of jaundice and the prevalence of antibody to hepatitis A, B, C, D, and E by age, sex, ethnic group, and geographical location
- c) Perform hepatitis B vaccine trials among the Belize Defence Force to investigate ways to immunize soldiers using more cost-efficient methods

- d) Determine the prevalence of HIV and HTLV-1 infections in selected populations.
- e) Determine patterns of drug resistance of *Neisseria gonorrhea* in various regions.
- f) Investigate the etiology and epidemiology of leishmanial infections
- g) Investigate illness from tick-borne disease
- h) Address epidemiologic targets of opportunity; causes of febrile illnesses in British Forces Belize (BFB), U.S. Army Corps of Engineers, etc.
-) Determine the sero-epidemiology of Chagas' Disease in Belize
- j) Determine patterns of malaria transmission; maintain surveillance for chloroquine-resistant *Plasmodium falciparum*.
- k) Determine vectorial capacity of putative malaria vectors.
- L) Validate the remote sensing models developed for use in predicting temporal and spatial changes in malaria vector abundance in Mexico, in a second ecologically similar area (i.e., Belize).

Areas of Research in Belize

Febrile Illnesses

Little was known about infectious diseases specific to Belize. Based on limited information from Belize and other Central American countries, it may be inferred that such diseases are common in Belize. Yellow fever has been reported from the Yucatan [1], dengue and malaria are endemic in Belize, and cutaneous leishmaniasis, in almost epidemic numbers, has been reported in British troops stationed in Belize [2]. Cutaneous and visceral leishmaniasis have been reported in Honduras and Guatemala [3]. Leptospirosis, and Venezuelan equine and St. Louis Encephalitis have also been found in neighboring countries [4]. Enterically transmitted non-A, non-B hepatitis has recently been identified in Mexico [5].

The Statistics Department at the hospital reported yearly admissions over the past several years for enteric fever of 4 to 6 cases; jaundice, 60 cases; and fever of unknown cause, about 150 cases. A great deal remains to be done to determine the prevalence and incidence of tropical infectious diseases agents in Belize.

To further define the prevalence of arthropod borne illness in certain groups in Belize, serologic testing for arthropod-borne illnesses was conducted at Ft. Detrich. Four Belizean populations were studied: Immigrant workers and families living and working on a banana plantation in Stann Creek district, workers at a Mayan excavation site at Caracol in Cayo district, persons of Spanish descent living in three villages in Cayo district who had blood drawn for a study of malaria, and persons admitted to the hospital in Belize or Cayo districts for non-malarious fevers.

Etiology of Jaundice and the prevalence of hepatitis A, B, C, D and E

In May 1991, concern that cholera may be spreading north through Central America prompted officials of the Belizean MOH to initiate an active search for possible cases. In the Cowpen area of southeast Belize there were no reports of illness resembling cholera, but banana farm workers and local health officials were concerned about the large number of people who were or had been ill with hepatitis. In some cases people had diagnosed themselves as having hepatitis, based upon the occurrence of jaundice. For others, the diagnosis had been made by local health professionals.

Cowpen residents considered the appearance of hepatitis to be an annual occurrence, associated with the dry season, and attributed the disease to the poor quality of water available. However, many believed the 1991 dry season was more severe and longer than usual and the numbers of cases, particularly in young adults, was greater than they had seen before. Concern about hepatitis increased when the death of a young, pregnant woman and her child were attributed to the disease.

The Belizean MOH responded to the apparent increase in hepatitis cases by requesting assistance from the Belize -United States Epidemiologic Research Center (ERC) in Belize City. In collaboration with the Uniformed Services University of the Health Sciences (USUHS), and with laboratory support from the Walter Reed Army Institute of Research (WRAIR), a preliminary investigation was done in May 1991 to try to establish a diagnosis. The May investigation by the Belizean team established the presence of hepatitis in the Cowpen area and the need for further study. This was followed in June 1991 by an expanded effort to identify and define cases and to do a cross-sectional study of hepatitis markers and related variables in banana farm workers and their families.

In order to better characterize the epidemiology of hepatitis viruses in Belize, we conducted three serologic prevalence studies. These were conducted among the Belize Defence Force in 1993; pregnant women in 1994; health care workers, 1994, and school aged children in 1995. In addition, we did intensive surveillance for cases of jaundice in the Stann Creek District during 1994 and 1995. With the results of these studies, we better characterized the problem of hepatitis, especially hepatitis B.

Hepatitis B vaccine trials

Hepatitis B is a common infection in Belize. It is also common in the US military, especially in Korea. Two obstacles to hepatitis B immunization prevent its widespread use. First, it is very expensive with a cost of approximately \$92 per person for full dose immunization. Second, immunization requires three doses given over a 6 month period. One way that hepatitis B vaccine has been administered at a lower cost is to administer Recombivax HB (MSD) at one-half the recommended dose (5 µg in 0.5 ml) in those <30 years. The other hepatitis B vaccine (Engerix B, SmithKline) has not been investigated at the lower dose of 5 or 10 µg. Use of Engerix at this lower dose would result in significant

cost savings. Therefore we conducted a vaccine trial among members of the Belize Defence Force which compared 5 or 10 µg doses of Recombivax or Engerix.

Second, , a fewer number of doses, i.e. 2 rather than 3 would improve the compliance rate with hepatitis B vaccine. In addition, giving all doses over a 14-16 week period (the duration of basic training and an A school) would mitigate the problem of giving vaccine at a new duty station or trying to give a dose when military personnel are in the field or underway. Therefore, we conducted a trial comparing two doses of 10 µg with three doses of 5 µg of Recombivax given over an 8-16 week period.

HIV Infection

HIV is prevalent throughout the world and HTLV-1 is common in some Caribbean areas. In order to estimate the extent of the problem in Belize, several studies were done. One involved screening blood bank samples by ELISA. This was initiated in Belize. Confirmation was done by US military testing during the early part of the grant. Later, confirmation was done by the Commonwealth Caribbean reference center or through PAHO. A second study was conducted among pregnant women in Belize, a study that also focused on hepatitis B and syphilis in this population. Finally, Dr. Michael Pitts, as part of his MPH degree from USUHS summarized the status of HIV infection in Belize in 1991.

Determine rates of resistance of *N. gonorrhea*

Resistance of *N. gonorrhea* to tetracycline and penicillin create difficulties in treating gonococcal infections. To determine rates of infection in Belize, Dr. Robert Kadlec collected isolates of gonorrhea, recorded the determination of susceptibility as performed in Belize, and transported some isolates back to the US for additional study of auxotype and serovar. This was part of his Masters in Tropical Medicine and Hygiene course.

Outbreak of Leishmaniasis in Belize Defence Force

Leishmaniasis is endemic in Belize. *L. mexicana* causes cutaneous lesions. In October, 1989, a team from USUHS, and two scientists from WRAIR (LTC Pete Perkins, entomologist and Col Jonathan Berman, clinician) investigated an outbreak of skin lesions among members of the Belize Defence Force. A total of 56 needle aspirates were acquired from 15 BDF and 2 civilian cases. Weather, epidemiologic and entomologic data were collected

Investigate Tick Borne disease

Workers at the ERC were asked to investigate the possibility of tick-borne diseases such as Lyme disease. A number of workers at the recently discovered Mayan ruin at Caracol were complaining of tick bites. A serologic survey of workers was performed.

Epidemiologic targets of opportunity

Because troops can become ill from a wide variety of agents when exposed in the field in the tropics, disease surveillance was conducted among US reserve units in Belize and among British marines in Punta Gorda in the south. This surveillance consisted of pre- and post-serologic testing for a number of viral agents. Some of the testing was done at Ft. Detrich. Post deployment serologic testing on British Marines was performed in England.

Seroepidemiology of Chagas' Disease in Belize

Chagas' disease is caused by *Trypanosoma cruzi*, and is transmitted by Triatoma species. Little is known about Chagas' disease in Belize. The British did some vector collections [6, 7] and one serologic survey in Belize in the mid 1960's [8]. To determine the potential for blood transfusion transmission, the prevalence of antibody to *T. cruzi* in blood donors was studied. In addition, sera from the Belize Defence Force was studied. Finally, sera from immigrant workers in the banana plantations was studied.

Field studies of malaria

Malaria is a major problem in Belize and other Central American countries, and it is growing. The Belize physician who was in charge of malaria for several years was a graduate of the USUHS MPH program. In 1994, approximately 10,000 cases of malaria were reported in Belize, a country of only 200,000 people. Approximately 95% of cases of malaria in Belize are caused by *P. vivax*. Recently, *P. falciparum* has become more common than previously. Several of our studies have involved

laboratory evaluation for malaria. These have helped determine the role malaria plays in febrile illness, anemia, and jaundice. Malaria films were made as part of a study in 1989 in a field evaluation by Dr. Peter Warfe, an MTM&H candidate. In addition, malaria smears have been made on patients presenting with clinical jaundice, and on school aged children in Stann Creek district.

In addition, 1994 Social Correlates of Malaria in Red Bank, Nancy Everett, RN, MPH, Shilpa Hakre, and Ruth Jaramillo conducted a questionnaire survey in 1994 in a village called Red Bank. This village of Mayan Indians was experiencing a very high rate of malaria each year (300/1000). The objective of the survey was to try to understand why this community was experiencing such a high rate of malaria and whether the people were being exposed at work in the banana plantations or in the village.

Studies of diarrheal diseases

During the study period, investigators at the ERC were asked to investigate the etiology of diarrheal disease. Some studies were done at a clinic in Belize City (Matron Roberts) and others were done in a small village. In addition, Belize also experience cases of cholera beginning in 1992. Since 1991, ERC staff were members of the cholera committee which investigated and made recommendations for the prevention, diagnosis and treatment of cholera.

Correlates of Premature infants in Belize

In 1992, Mohan Kishore, MBBS, MPH, as part of his MPH curriculum, studied correlates of prematurity among babies born at the Belize City Hospital.

Studies of vectors of malaria

Because malaria has increased in incidence rapidly and because little is known of the vectors of malaria, entomological studies have been performed in Belize. Some of these have dealt with mosquito systematics, geological and environmental determinates of Anophele larvae, types of malaria vectors in both northern and southern Belize, genetic structure of Anopholes in Belize compared with other countries, and the use of remote sensing to identify the location of malaria vectors and subsequently, areas at highest risk for malaria

Studies from Pakistan

Hepatitis E in Pakistan

During this period of time, work with collaborators at the Pakistan United States Laboratory for Seroepidemiology has been proceeding slowly but with some important accomplishments. No American investigator has visited Pakistan in the past 5 years. However, work on hepatitis E, an enterically transmitted non-A, non-B hepatitis has continued in the USA on specimens brought from Pakistan.

Hepatitis E is an important cause of jaundice in southwest Asia. Jaundice is a common problem in the military in Pakistan. Studies we conducted in 1987-89 indicate that hepatitis B causes about one-third of cases admitted to the Combined Military Hospital in Rawalpindi while two-thirds of cases are non-A, non-B cases [9]. In addition, epidemics of hepatitis have occurred in military settings in Quetta, Mardan, Sargodha, Abbottabad and in Rawalpindi [10, 11].

Arthropod-borne viral infections in Pakistan

Sera from three populations in the Pakistan Army were studied at Ft. Detrich by LTC Ksiasak and LTC LeDuc for antibody to arthropod-borne viral infections as well as leptospirosis. The populations were recruits, students at a military high school who

were involved in an outbreak of hepatitis E, and patients with febrile illnesses admitted to the Combined Military Hospital in Rawalpindi.

BODY

Research in Belize

Febrile Illnesses

Cases of unexplained fever were selected by trained Belizean collaborators from patients over 12 years of age presenting at the Belize City, San Ignacio and Orange Walk Hospitals. Patients with sickle cell disease, meningitis, dysentery, or evidence of peritonitis, wound infection, pneumonia, tuberculosis or HIV infection were not included in the study. Thick and thin malaria films were prepared from finger sticks and examined. Patients positive for malaria were listed but not studied. Patients selected for the study were divided into two groups, those with and those without jaundice. A systematic clinical exam was performed and blood was obtained for diagnostic tests. Sera were analyzed for the presence of antibodies to arthropod-borne viruses and leptospirosis. Based on results provided by James W. LeDuc, Ph.D. in 1991, some 61 "convalescent" sera were tested. None were reactive for Chagres virus, 15 reactive to St. Louis encephalitis virus, 7 for Eastern Equine Encephalitis and 16 for Venezuelan encephalitis, 16 for dengue, 4 for Ft. Sherman virus, and 5 for leptospirosis. Results of virus isolation from acute sera are unknown at this time.

Studies of 392 immigrant workers (mostly from Honduras, Guatemala, and El Salvador at Cowpen indicated antibody to Leptospirosis in 18.6%, Dengue 2 in 12%, St. Louis Encephalitis in 22%, Venezuelan Equine. Encephalitis. in 36%, with 1-15% reactive to other viruses tested (Table). Similar patterns were observed in workers at Coracol. Persons with fever and those in a malaria survey had a somewhat lower prevalence to Dengue 2.

Etiologies of jaundice and prevalence of hepatitis A, B, C, D and E

Cowpen Study

The results of the investigation in 1991 in the Cowpen area revealed a very high prevalence (about 70%) of hepatitis B markers among both children and adults in the communities surrounding the banana plantations. The results of this study are summarized in abstract #3 and have been published [12]. No evidence for hepatitis D or E was found among these immigrant workers.

Incidence and Etiology of hepatitis in Stann Creek District

Because of continued cases of hepatitis in Stann Creek District, a prospective study of the etiologies of jaundice in Stann Creek District was conducted during 1994. Of approximately 50 cases of jaundice which have been analyzed to date, approximately 35 were caused by acute hepatitis B, 4 by acute hepatitis A, and others were associated with malaria or are still under investigation. The highest incidence rates have been found in the communities of immigrant workers around the banana plantations. Most cases are among young adult men, frequently single, though a number of women, including pregnant women have been studied. Mayan Indians living and working in the area also have a high incidence. Predominant modes of transmission have not been identified.

Prevalence of hepatitis viruses in the Belize Defence Force

Additional studies to define the prevalence of hepatitis viruses in Belize have been completed and published [13]. The prevalence of hepatitis A, B, and C among members of the Belize Defense Force was studied. Overall, approximately 90% of members had been exposed to hepatitis A, 35% to hepatitis B, and <1% to hepatitis C (see Abstract 4).

Prevalence of hepatitis B, HIV and syphilis in pregnant women

A prevalence study of pregnant women was headed by Dr. Alicia Scott-Wright. Among 548 women studied, approximately 15% had been exposed to hepatitis B. The

prevalence was much higher in women of Garifuna and Creole ethnic groups than among Mestizo, Mayan, East Indian or Dutch German-Mennonite groups. Antibody to syphilis and hepatitis B were epidemiologically associated.

Prevalence of Hepatitis B in Health Care Workers

A study of the prevalence of hepatitis B among health care workers (HCW) was carried out among health care workers in all 6 districts of Belize. Overall, approximately 30% of HCW had evidence of hepatitis B (Abstract #7). As with studies in the Belize Defence Force and pregnant women, hepatitis B exposure was associated with increasing age, ethnicity, district of residence. Hepatitis B exposure was not more common in health care workers who reported needlestick injuries.

Prevalence of hepatitis B in school-aged children

Finally, a study of the prevalence of hepatitis B was conducted among school-aged children in Stann Creek District. These results indicated the highest rates of antibody to hepatitis B were found among children in the Mayan and Spanish immigrant communities near the banana plantations (about 65%). Lower rates were found among grade school children in an urban community (9%). Rates among high school aged children ranged from 36%-42%.

These studies of the incidence of hepatitis in Stann Creek district and the prevalence of hepatitis B among soldiers, pregnant women, health care workers, and children paved the way for a rational approach for hepatitis B control. Hepatitis B vaccination has been initiated among members of the Belize Defence Force with almost all susceptible member immunized in one of two vaccine trials we conducted (see below). In addition, Dr. Joe Bryan worked with Dr. Polanco, the head of epidemiology in the Ministry of Health and Nurse Moguel to develop a program for Stann Creek District and Health Care workers. Therefore, the studies on the etiology, incidence and prevalence of hepatitis have resulted in the implementation of control programs.

Hepatitis B vaccine Trials

As indicated in abstract #8 and soon to be published [14], approximately 275 personnel of the Belize Defence Force were evaluable in a trial comparing three doses of 5 or 10 µg doses of Recombivax or Engerix. Vaccine for this trial was purchased through the Military Liaison Office in Belize and was not charged to the grant. In this trial, Recombivax regimens performed better than Engerix regimens. The study confirms the use of 5 µg doses of Recombivax in this age group. The results with Engerix suggest these low doses might have some usefulness in controlling hepatitis B; however, the seroconversion rates and antibody titers were lower than among Recombivax regimens.

A second hepatitis B trial compared Recombivax 5 µg in 0.5 ml at 0, 1, and 2 months with Recombivax 10 µg in 1.0 ml at 0 and 2 months. Vaccine for this trial was purchased through USUHS, not charged to the grant. No results of this trial are presently available.

HIV infection

No data are presently available on the prevalence of HIV in blood donors in Belize. This has all been taken over by the Ministry of Health.

In 1991, Dr. Pitts reported on 43 cases of AIDS in Belize. It was reported that 49% were felt to be heterosexual, 29% Homo- or bisexual, and 7% perinatal transmission (report attached). By the end of 1994, approximately 100 persons in Belize had been diagnosed with AIDS (verbal communication to Dr. J. Bryan by Dr. Pitts).

Among approximately 550 pregnant women studied in Belize in 1993 (abstract # 6), only one was found to have antibody to HIV by ELISA. This sera has not been confirmed by western blot. Since it has been shown that perinatal transmission can be decreased when a pregnant mother receives an antiretroviral agent such as AZT [15], routine screening of pregnant women has been recommended.

No data is available on rates of HTLV-1 in Belize. This is an area for ripe investigation since HTLV-1 associated paraparesis and leukemia have been reported from other Caribbean countries.

Determine rates of resistance of *N. gonorrhea*

Dr. Robert Kadlec studied *N. gonorrhea* in Belize as part of his MTM&H degree. Between Oct 88 and June, 1989, 56 culture proven cases of gonorrhea were recorded by Central Medical Lab during the study period. When studied by disc diffusion methods, the majority were found to be resistant to penicillin (40/56; 71%). Two of 40 (5%) were reported as resistant to penicillin and tetracycline.

Seven isolates were brought to the US. Six of the seven had a penicillin MIC of ≥ 2.0 and five of 7 were resistant to tetracycline MIC ≥ 2 . All were beta lactamase negative suggesting a chromosomal mediated resistance. All were susceptible to ceftriaxone and spectinomycin. The primary auxotypes were pro or proto and the serovars mainly AB1 and 1B5.

Considerable work needs to be done to monitor drug resistance by the gonococcus in Belize to drugs such as ceftriaxone, spectinomycin, and quinolones.

Outbreak of Leishmaniasis in Belize Defence Force

Among persons studied, *L. mexicana* was obtained from at least one person's lesions. Some persons had been started on treatment with paromomycin. Lack of insect repellent while on patrol at the summer training camp in Cayo district was a risk factor for infection. (See abstract 2). Dr. Craig, the Belize Defence Force surgeon and other physicians in Belize have recently described the use of topical paromomycin for the treatment of cutaneous leishmaniasis [16].

Tick-borne fever

Analysis of sera from the 47 workmen at the Caracol archeological site was undertaken by the USAMRIID lab. Preliminary results showed 7 of the 47 positive for Lyme disease. However, two of these specimens are also positive for syphilis, a known cross reactor. Antibodies to other infectious diseases will cross react in this enzyme linked immunosorbent assay (ELISA); therefore, positive results with *Borrelia burgdorferi* need to be confirmed with other diagnostic tests.

Epidemiologic targets of opportunity

Approximately 600 British Royal Marines were deployed for 6 months to Rideau camp in southern Belize in 1993. Sera were collected on these British Forces before and after deployment. In the summer of 1993, sera on men who unexplained illnesses were collected by Surgeon Lt. Simon Leigh-Smith and were sent by CDR Joe Bryan to Major Korch at Ft. Detrich. There were sera on 9 men, four with paired sera. No definite diagnosis of acute disease could be identified in studies for Punta Toro, Ft. Sherman, VEE, vesicular stomatitis, St. Louis encephalitis, Changuinola, Chagres, dengue 2, yellow fever, Oropouche, and Hantaan. Several of the sera had antibody to *Leptospira* and most had antibody to yellow fever, probably as a result of immunization. No results of studies on these men which were conducted by Dr. Lloyd Graham in England are available at this time.

Seroepidemiology of Chagas' Disease in Belize

Results of the serologic testing are found in Abstract 5. No sera from approximately 500 members of the Belize Defence Force were reactive, <1% of approximately 900 blood donors were reactive, but approximately 5% of 400 immigrant farm workers had antibody. It is not known if these persons were infected in Belize or if they were

infected in their home countries of Guatemala, Honduras, and El Salvador before coming to Belize.

Field studies of malaria

A household survey of malaria was conducted by Dr. Peter Warfe and Mr. Fred Duncan among villagers in a rural area of Belize in 1989. Venous blood samples were obtained from 186 persons over the age of 12 years in the villages of Bullet Tree, San Antonio and Las Flores in Cayo District near the Guatemalan border. Few pregnant women were studied. Thick smears were studied by an experienced microscopist and the QBC tube method was used by another investigator. *P. vivax* was observed in 6 samples for a prevalence of 3.2%.

in 1994, blood films for malaria were obtained as part of an evaluation for clinical jaundice. Approximately 5 of 50 persons with jaundice had malaria parasites observed, usually *P. falciparum*. Most of these persons were from the southern part of Stann Creek district and were either Mayan Indians or immigrants from other Central American countries.

As part of the school survey for hepatitis markers, malaria smears were read. No malaria parasites were observed in students in town. However, five of approximately 200 children in rural schools near banana plantations were infected with malaria, three with *P. falciparum*. These results were given to malaria control personnel so that patients could be treated.

The questionnaire survey conducted by Nancy Everett RN, MPH revealed that malaria was of major concern to the villagers in Red Bank, that diagnostic results were frequently delayed in being returned because of transportation difficulties, that follow through with treatment was often inadequate, and that housing was inadequate to prevent mosquito entry.

Studies of diarrheal diseases

Studies of diarrhea in 1991 in Orange Walk and Cayo district and on two cayes (Abstract # 1). The incidence of diarrhea was highest in January through March. Some of the stools were reactive for rotavirus.

Another investigation of diarrhea was carried out in February 1994 at Crooked Tree in Orange Walk District by ERC team members. Several adults were ill with diarrhea, high fever and abdominal cramps and in one person, bloody diarrhea. Approximately 15 children from the school were ill. Stool samples were obtained from 5 children and 2 adults. In one adult, the sample was reactive for rotavirus and also grew *Shigella flexneri*. Studies for Salmonella, Shigella, Vibrio and rotavirus were negative on the others. Some samples on patients hospitalized in Belize City with vomiting and diarrhea were also reactive for rotavirus. Therefore, it appears that rotavirus is a cause of diarrheal illness in Belize, especially in the winter months, similar to the US and Mexico.

Members of the ERC also investigated some of the first cases of cholera in Belize and helped train lab workers in the diagnosis of cholera. The number of cases of cholera reported in Belize was 0 in 1991, 159 in 1992, 135 in 1993 and 2 up through September 1994 (MMWR, 1994). Continued surveillance is clearly indicated.

Correlates of Premature infants in Belize

The major findings of the case-control study identified infants of mothers of Mayan ethnicity, family income of < \$7199/year, < 4 prenatal visits, and medical conditions during pregnancy to be associated with infants < 2500 grams.

Studies of vectors of malaria

Studies of the larvae and adult Anophelene mosquitoes have been some of the most instructive studies carried out through the ERC (abstracts 9-18). Vectors of malaria have been characterized in both northern and southern Belize. Genetic and morphologic studies have been carried out, and most interesting, areas at highest risk for malaria have been determined by remote sensing. This has led to a new grant which began in Belize this spring. This new grant will focus on malaria control using remotely sensed data to predict malaria transmission.

Studies in Pakistan

Studies on Hepatitis E in Pakistan

The outbreaks of hepatitis in Pakistan that have been studied most extensively are those which occurred in Sargodha and Abbottabad in 1986 and 1987, respectively. The outbreak in Sargodha was investigated by Dr. Iqbal, a graduate of the USUHS MPH program. Over 100 men were hospitalized in epidemic of non-A, non-B hepatitis [11]. Fecal and serum specimens were carried to the US and studied at the NIH in collaboration with Dr. Robert Purcell.

Fecal specimens from patients in the Sargodha outbreak were transported to the US and studied by Immune Electron Microscopy by Dr. Ticehurst and coworkers. The pattern of fecal excretion was studied initially with this labor intensive technique [17]. HEV was visualized most commonly in specimens obtained within two weeks of the onset of jaundice. In most specimens, hepatitis E was difficult to visualize. However, in one specimen from patient #55 (SAR 55), virus was more common.

Fecal suspensions of SAR 55 injected into non-human primates and resulted in hepatitis and abundant excretion of hepatitis E. The genome of this isolate was sequenced [18]. When portions of this genome were expressed in baculovirus, it was found that the proteins reacted with antibody in patients' sera from the outbreak resulting in a diagnostic test [19]. This diagnostic test was used to further characterize the IgM and IgG anti-HEV response in patients with hepatitis in the Sargodha outbreak [20, 21]. These studies indicated that IgG and IgM anti-HEV are both present in over 90% of patients at the time of admission. The IgG anti-HEV persists, at slowly decreasing titers, for at least 18 months; however, IgM anti-HEV persists for only about 4 months. The Sar 55 sample has also been evaluated in terms of infectivity to non-human primates. Oral administration of a suspension does not result in hepatitis; however intravenous administration does [22].

Proteins expressed from this genetic sequencing have also been used to induce active immunity to hepatitis E in animals [23]. Immune plasma also will protect animals from HEV infection. Pre existing antibody to HEV appeared to protect men who were exposed in the Sargodha and Abbottabad outbreaks from illness [21]. Evidence that antibody against HEV, whether derived passively, actively through prior infection, or through active immunization with recombinant products protects against HEV lay an important groundwork for a vaccine against hepatitis E.

Another outbreak which occurred in Abbottabad, Pakistan in 1987 has also been investigated. Approximately 150 personnel were hospitalized in the outbreak. Like the outbreak in Sargodha, the source of contamination appeared to be from a sewer leakage into water pipes. An ELISA produced by Genelabs was used to detect anti-HEV in some of the serum specimens [24]. In addition, using primers supplied by NIH, affinity capture PCR was used to identify HEV in feces in patients with clinical hepatitis E [25].

Later, primers from Genelabs were used to more completely characterize HEV in feces of patients in the Abbottabad outbreak using the affinity capture PCR. Some fecal specimens were collected serially from 13 patients and others were only single specimens (See abstract #20). The results of the pattern of fecal excretion in feces using PCR were similar to those observed with IEM; however, PCR detected HEV more frequently and for at least 4 weeks after onset of jaundice in some patients. In addition, serologic studies using the serologic test developed by Tsavev have elucidated the pattern of anti-HEV in these patients. This was most thoroughly studied in 44 patients from whom three serum specimens were collected over 4 months (see abstract #20).

Studies on arthropod borne illnesses in Pakistan

Serologic studies of the prevalence of arthropod borne illnesses among persons with hepatitis have been completed and accepted for publication [26]. These indicate

that about one-third have antibody to sand fly fever viruses and West Nile virus, 25% have antibody that reacts to Japanese encephalitis virus, and none reactive for Haantan or Crimean Hemorrhagic Fever viruses.

CONCLUSIONS

The past five years have been immensely productive in studying important illnesses in both Belize and Pakistan. In the area of defining etiologies of febrile illness, especially arthropod borne illness, some information has been obtained from studies of immigrant workers in the banana growing area, persons admitted to the hospitals, and American and British Forces in Belize. What is needed in Belize is better laboratory capability in the country to diagnose acute dengue, and conduct surveillance for other arthropod-borne diseases. The practice of shipping specimens to Ft. Detrich or even to labs in Guatemala or a Caribbean center is cumbersome, time consuming, and results are frequently delayed in being returned. Much work remains in the area of defining clinical illness caused by viruses transmitted by arthropods.

In Belize, the most complete evaluation of a class of illness has been among hepatitis viruses. Studies that represent nation-wide surveys of hepatitis viruses have been conducted among the Belize Defence Force, health care workers, and pregnant women. Based on these surveys that indicated the highest prevalence of hepatitis B to be in Stann Creek district, prevalence studies in this district of immigrant farm workers and school aged children were conducted. Finally, a difficult, year-long study of active surveillance for acute cases of jaundice in Stann Creek district yielded information about the incidence and some risk factors for hepatitis viruses. The information from these studies is now being formulated into a hepatitis B vaccine policy in Belize.

Immunization against hepatitis B has been ongoing in the Belize Defence Force since 1992 resulting in one of the few militaries in the world to be almost completely immunized against hepatitis B. This has resulted from two vaccine trials conducted in collaboration with the physician for the Belize Defence Force, Dr. Craig. The first vaccine trial, a comparison of the two recombinant vaccines, resulted in confirmation that 5- μ g doses of Recombivax produce excellent antibody responses, but that lower

than recommended doses of Engerix B performed less satisfactorily and should not be recommended. Continued immunization of incoming military recruits should be encouraged and future studies may well be possible.

In the area of HIV and sexually transmitted diseases, good progress has been made in the initiation of screening blood bank specimens for HIV. The major mode of transmission in Belize is presently heterosexual with some homosexual-bisexual transmission. The epidemiology should continue to be monitored because of the increased use and trafficking of illegal drugs in Belize. Work in sexually transmitted disease clinics should identify the etiologies of STD's, monitor the antibiotic susceptibility patterns, treat STD's, especially those that cause genital ulcers because of their potential to spread HIV and Hepatitis B, and to monitor rates HIV among those attending the clinics.

Leishmaniasis appears to be an illness that may be increasing in incidence. Belize is commonly sited by travelers who develop leishmaniasis [27]Dr. Craig of the BDF has taken a special interest in this illness, partly because of the number of cases occurring among members of the Belize Defence Force. He also attends a large number of persons who come to him for advice and medication. Areas that need additional work include defining the epidemiology, culture and histology diagnosis of the organisms, speculating the organisms, and doing additional drug trials for treatment. Trials with topical paromomycin look encouraging, but work on the best vehicle and other formulation issues still need attention. Better treatment for mucocutaneous leishmaniasis also is required.

In addition to leishmania which is transmitted by sand flies, additional work needs to be done on the ecology of ticks and the diseases they may transmit in Belize. The work that was done among workers at the Carocol Mayan excavation site were tantalizing, but need confirmation and additional work. Other vector borne diseases such as Chagas' Disease need attention to the habitat of the vector. No work on the

ecology of reduviid bugs has been done since the British did preliminary work in the mid 1960's. The issue of whether *T. cruzi* is being transmitted in Belize needs to be resolved by studies of potential cases, longitudinal studies of populations at risk, and continued monitoring of the blood supply.

Entomologic studies have revealed much about vectors of malaria in Belize. Studies of the taxonomy of *Anopheles darlingii* revealed important findings that relate to identification of malaria vectors. Environmental studies indicate that malaria vectors breed among naturally occurring mats of filamentous algae and cyanobacterial mats in northern Belize. Studies in the south of Belize indicate that *An. vestitipennis* and *albimanus* now are commonly found in homes; in the 1940's *An. darlingii* was more commonly found. Later studies indicated that *An. darlingi* is a riverine mosquito that oviposits in highly selected habitats, i.e. shade and submersed vegetation but that *An. albimanus* larvae are found in association with algae. Comparison of the genetics of mosquitoes found in Belize with those found in other areas of Central and South America have also been carried out.

Perhaps most exciting has been the demonstration that multispectral satellite data can be used to predict areas at highest risk for malaria. These findings are now being utilized to try to control malaria by focusing manpower and resources on areas at greatest risk.

The work in Pakistan has resulted in exciting work on hepatitis E. The epidemiology, the diagnosis, the pattern of antibody and the pattern of excretion in stool have now been studied, in part because of new technology that has resulted from reagents found in specimens brought from Pakistan. The next phase of work is likely to center on additional studies of epidemiology (why are young adults susceptible to an enteric infection when most are immune to hepatitis A), pathology (why do pregnant women have a high mortality with hepatitis E), and development of a vaccine for hepatitis E.

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ADDENDUM

List of Abstracts and presentations

Belize and Pakistan

1. **Outbreak Of Rotaviral Gastroenteritis In Belize.** Krieg RE, Lopez JA, Reyes LG, Jaramillo R, Duncan F. Presented as a poster at the 37th Commonwealth Caribbean Medical Research Council, Curacao, Netherlands Antilles, 22-25 April, 1992.
Also presented at the annual meeting of the Society of Armed Forces Medical Laboratory Scientists, San Antonio, 1992.
2. **An Outbreak Of Cutaneous Leishmaniasis In The Belizean Defence Forces, Belize, C.A.** Craig P, Krieg RE, Brady WE, Perkins PV, Berman JD, Duncan JF, Legters LJ.
Presented at the American Society of Tropical Medicine and Hygiene, New Orleans, LA, 1990 and the Xth Medical and Dental Congress, Belize City, Belize, 1991.
3. **Initial Report Of A Hepatitis Investigation In Rural Belize.** Hoffman KJ, Gaydos JC, Krieg RE, Duncan JF, MacArthy PO, Ticehurst JR, Jaramillo R, Reyes LG, Sjogren MH, Legters LJ. Transactions of the Royal Society of Tropical Medicine and Hygiene 87:259-262, 1992.
Presented at Prevention 93 in Atlanta, GA, April 17-20, 1993 and at the 37th Annual Meeting of the Commonwealth Caribbean Medical Research Council in Curacao, Netherlands Antilles, 22-25 April, 1992.
4. **The Prevalence Of Hepatitis A, B And C Among Different Ethnic Groups In Belize.**
Craig PG, Bryan JP, Miller RE, Reyes L, Hakre S, Jaramillo R, Krieg RE. Am J. Trop Medicine and Hyg. 49:430-434, 1993.
Presented at the 38th Annual Commonwealth Caribbean Medical Research Council Meeting, Apr 21-24, 1993, Trinidad. This resulted from masters thesis research for Dr. Peter Craig.
5. **Testing for Chagas Disease in 3 Populations in Belize.**
Jaramillo J, Gabourel I, Bryan JP.
Presented at the Belize Medical and Dental Association Meeting, September 1993 Belize City, at the American Society for Microbiology Meeting in Los Vegas, April, 94 and at the American Society for Tropical Medicine and Hygiene, Cincinnati, OH, Nov. 1994.

6. **Seroprevalence Of Hepatitis B, Syphilis And HIV-1 In Women Attending Prenatal Clinics In Belize.**
 Scott-Wright A, Hakre S, Bryan JP, Jaramillo R, Reyes L, Cruess D, MacArthy P, Gaydos J. A masters thesis for Alicia Scott-Wright, MD conducted during the summer of 1993.
 Presented at the Commonwealth Caribbean Research Council Meeting April, 1994 and submitted for publication.
7. **Seroprevalence of hepatitis B Markers in Health-Care Workers in Belize.** Hakre S, Reyes L, Bryan JP.
 Presented at the annual meeting of the American Society for Tropical Medicine and Hygiene, Cincinnati, OH, November 94. Am Soc Trop Med Hyg: In Press
8. **Randomized Comparison Of 5 And 10 µg Doses Of Two Recombinant Hepatitis B Vaccines.** Bryan JP, Craig PG, MacArthy P, Reyes L, Hakre S, Jaramillo R, Legters L. .
 Presented at the 33rd Annual Interscience Conference on Antimicrobial Agents and Chemotherapy. New Orleans, October 17-20, 1993, Abstract 665. Vaccine: In Press
9. **Variation In The Hindtarsal Markings Of *Anopheles Darlingi* (Diptera: Culicidae) In Belize.** Harbach RE, Roberts DR, Manguin S. 1993. Mosquito Systematics 25(3): 192-197.
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ABSTRACTS

GASTROENTERITIS OUTBREAK IN BELIZE

Richard E. Krieg, Ph.D., Jose A. Lopez, MD, MPH, Linda G. Reyes, BS, Ruth Jaramillo, BS, MT (ASCP) and J. Fred Duncan

ABSTRACT

An outbreak of gastroenteritis was identified in the Orange Walk, Cayo and Belize Districts of Belize, and in San Pedro, Ambergris Caye and Caye Chapel in February 1991. Forty-five of the 467 cases reported through mid-March were tested for the presence of rotavirus antigen (Pathfinder TM, Kallstead Diagnostics, Austin Texas) in fecal specimens. Of these, 23 (51%) were positive. The seasonal increase of gastroenteritis in the region is reported to occur during October and November; however, an analysis of 1605 reported cases of gastroenteritis since October 1988 at a clinic in Belize City, indicated that during this period, the seasonal increase in incidence occurred between January through May and that the peak incidence was from January through March. This seasonality coincides with the dry season in Belize, which supports the supposition that the mode of rotavirus transmission is fecal-oral. There was no significant difference in the sex distribution. Over 69% of the reported cases in the current outbreak and approximately 45% of the historical cases were in infants and children ≤ 4 years of age. The percentage of cases in individuals >4 years of age is higher than would be expected in an outbreak due solely to rotavirus, suggesting that other etiologies may have caused some cases.

Presented as a poster at the 37th Commonwealth Caribbean Medical Research Council, Curacao, 22-25 April, 1992. Also presented at the annual meeting of the Society of Armed Forces Medical Laboratory Scientists, San Antonio, 1992.

AN OUTBREAK OF CUTANEOUS LEISHMANIASIS IN THE BELIZEAN DEFENCE FORCE

Richard E. Krieg, Ph.D., William E. Brady, MPH, PA-C, Peter V. Perkins, Ph.D.,
Jonathan D. Berman, MD

ABSTRACT

On 23 October 1989, we were notified by the Defense Attaché, United States Embassy, Belize, Central America of an outbreak of cutaneous leishmaniasis in the Belizean Defence Force (BDF). We volunteered to assist and in response to requests from the BDF, the Ministry of Health (MOH), and the Defense Attaché an investigation team was formed and a protocol was developed and approved by the MOH and the Department of Research Administration, Uniformed Services University of the Health Sciences (USUHS).

The team consisted of Colonel Richard E. Krieg, Jr., Ph.D. (Team Chief); William E. Brady, MPH, PA-C (Epidemiologist); Lieutenant Colonel Peter V. Perkins, Ph.D. (Entomologist); and Colonel Jonathan A. Berman, MD. (Clinician). The team was in Belize, Central America 6-18 November 1989. During that time, 15 of 18 reported by the BDF Medical Officer were reviewed. Histories were obtained and needle aspirates of lesions and a blood specimen were taken for cultures, fluorescent antibody stains, and antibody studies.

The BDF patient data was collected at Price Barracks, Ladyville. The British Forces, Belize data were obtained at the Airport Camp Hospital. The two civilian cases seen were both from the Cayo District. There was photographic documentation of all lesions. The meteorologic data were provided by the weather station at the Belize City Airport. Epidemiologic and entomologic data were collected throughout the Cayo District and are detailed in the reports of those two investigators.

A total of 56 needle aspirates cultures were obtained from the 15 BDF and 2 civilian cases. The laboratory tests have not been completed. To date, only one culture has grown; but, it is premature to consider the others negative. The direct fluorescent antibody stains have not yet been completed. The speciation of the sandflies has been started.

The epidemiologic data indicate that a combination of factors were probably responsible for the observed outbreak. The unusually dry months of May and June enhanced sandfly survival and increased risk of exposure. The lack of repellent and/or lack of use of repellent by the BDF forces while on patrol and at Summer Camp were risk factors. Summer camp was in July in an area of Cayo that is endemic for leishmaniasis.

Recommendations for continued surveillance, therapeutic regimens, preventive measures, and entomologic studies are included.

Presented at the American Society of Tropical Medicine and Hygiene, New Orleans, LA, 1990 and the Xth Medical and Dental Congress, Belize City, Belize, 1991.

INITIAL REPORT OF A HEPATITIS INVESTIGATION IN RURAL BELIZE

Kenneth J. Hoffman, MD, MPH, Joel C. Gaydos, MD, MPH, Richard E. Krieg, Ph.D., J. Fred Duncan, Jr., Philip O. MacArthy, Ph.D., John R. Ticehurst, MD, Ruth Jaramillo, BS, Linda G. Reyes, BS, Maria H. Sjogren, MD, MPH, and Llewellyn J. Legters, MD, MPH

ABSTRACT

In spring 1991, Belizean health officials expressed concern about a possible hepatitis outbreak in a banana farming district. A study was designed to identify cases and to address the serologic prevalence of hepatitis virus markers. Three populations were studied: 1) anyone meeting a clinical case definition for hepatitis, 2) any designated banana worker, and 3) a random sample of households in the community. Information was collected using questionnaires, and sera were drawn for laboratory testing. This report presents the preliminary results of a study conducted in June 1991. Among people who met the clinical case definition, 24% of 42 tested had IgM antibody to hepatitis B virus (HBV) core antigen (anti-HBc IgM). In the worker and household survey populations, 284 and 280 people, respectively, were tested for anti-HBc IgM. In each group, 4% were positive. HBV surface antigen was found in 37% of clinical cases (43 people were tested), 18% of workers, and 13% of people in the household survey. Among the three study populations, the prevalence of HBV core antibody (anti-HBc) ranged from 73% to 81%. Almost all tested had evidence of prior hepatitis A virus infection. Evidence of prior infection with hepatitis viruses A and B was widespread, but an etiology could not be established for most of the clinical cases. However, the prevalence of hepatitis B markers in this population was very high compared to other reports from the Caribbean.

Presented at Prevention 93 in Atlanta, GA, April 17-200, 1993 and at the 37th Annual Meeting of the CCMRC in Curacao, Netherlands Antilles, 22-25 April, 1992.

Published in the Transactions of the Royal Society of Tropical Medicine and Hygiene 87:259-262, 1992.

THE PREVALENCE OF HEPATITIS A, B, AND C INFECTION AMONG DIFFERENT ETHNIC GROUPS IN BELIZE

Peter G. Craig, MBBS, MTM&H, Joe P. Bryan, MD, Robert E. Miller, Ph.D., Linda Reyes, BS, Shilpa Hakre, BS, Ruth Jaramillo, BS, and Richard E. Krieg, Ph.D.

ABSTRACT

Little is known about the prevalence of infection with hepatitis viruses in Belize, Central America. We conducted a serologic survey among members of the Belize Defence Force (BDF), which is composed of the five major ethnic groups in Belize, to estimate prevalence rates of hepatitis A, B, and C among military-aged men and women in Belize. Of approximately 600 men and women in the BDF, 492 (82%) completed a questionnaire and blood collection. Antibody to hepatitis A was found in 94%, with similar rates by age, sex, rank, and ethnicity. Antibody to hepatitis B core antigen (anti-HBc) was found in 31%. Rates of anti-HBc varied significantly among the ethnic groups with the lowest rates in Mestizo (5%) and Mayan Indians (9%), and significantly higher rates among Creoles (30%) and Garifuna (56%). Rates increased with increasing age from 28% in those 18-24 years old to 35% in those ≥ 35 years old ($P=0.07$, by chi-square test for trend). Hepatitis B surface antigen was found in 21 (4%) overall. Antibody to hepatitis C was found in two (0.4%). In this young healthy population, exposure to hepatitis A before the age of 18 is almost universal, while exposure to hepatitis B is related to age and ethnic origin.

Presented at the 38th Annual Commonwealth Caribbean Medical Research Council Meeting, April 21-24, 1993, Trinidad.

Published in the American Journal Tropical Medicine and Hygiene. 49:430-434, 1993.

PREVALENCE OF ANTIBODY TO TRYPANOSOMA CRUZI **IN THREE POPULATIONS IN BELIZE**

Ruth Jaramillo, BS, MT, Joe P. Bryan, MD, Joan Schur , Alfred A. Pan, Ph.D.

ABSTRACT

A cross-sectional study was conducted to determine the prevalence of antibody to Trypanosoma cruzi, the agent of Chagas' disease among three populations in Belize. Specimens were tested using a second generation enzyme-linked immunoassay (EIA). Confirmatory testing with three Single-antigen EIAs and a radioimmunoprecipitation assay (RIPA) were performed. Eight (0.8%) of 932 blood donors at the Belize City Hospital were reactive including 4 (6%) of the 65 from countries known to be endemic for Chagas' disease and four (0.5%) of 831 from Belize. Among 467 healthy members of the Belize Defense Force, none were reactive. The third group included workers and families primarily from other Central American countries living at a banana plantation in a rural area of the country. Twenty-six (5.9%) of 442 sera were reactive. The age prevalence was 5.3% of 75 < 15 years, 4.2% of 236 15 to 34 years, and 9.7% of 124 > 35 years ($p < .05$ by chi-square for trend). The prevalence was similar in males (6.7% of 280) and females (5.8% of 154). The prevalence of those born in Belize 4/56 (7.1%) was similar to the prevalence of those born in El Salvador 9/110 (8.2%), Guatemala 5/117 (4.3%) and Honduras 8/129 (6.2%). Of the four persons with reactive sera who were born in Belize, the immigrant mother of one was also reactive suggesting possible congenital transmission. Among 30 sera repeatedly reactive by EIA to T. cruzi, 20 were reactive by at least 2 of 3 single antigen confirmatory EIA tests and 28 by RIPA. Additional studies should focus on the epidemiology of T. cruzi and ways to reduce risk of transfusion-related infections in Belize.

Presented at the Belize Medical and Dental Association Meeting, September 1993
Belize City, at the American Society for Microbiology Meeting in Los Vegas, April, 94
and at the American Society for Tropical Medicine and Hygiene, Cincinnati, OH, Nov.
1994.

PREVALENCE OF HEPATITIS B AND HIV-1 AMONG WOMEN ATTENDING PRENATAL CLINICS IN BELIZE

Alicia Scott-Wright, MD, MTM & H, Linda Reyes, BS, Shilpa Hakre, BS, Ruth Jaramillo, BS, David Cruess, Ph.D., Philip. MacArthy, Ph.D., Joel Gaydos, MD, MPH, Joe P. Bryan, MD

ABSTRACT

We initiated hepatitis B (HB) screening of women attending selected prenatal clinics in Belize. Risk factors for HB infection and demographic data were determined by interview. Of 548 women, 81 (15%) were seropositive for HB Core Antibody (anti-HBc); one had HB surface antigenemia. Antibodies to the HIV-1 were detected in one woman. Fifteen women had reactive syphilis serology. Anti-HBc seroprevalence varied by district (range 3%-44%) and by ethnicity (Creole, 19%; Garifuna, 43%; East Indian, 6%; Mayan, 22%; Mestizo/Spanish, 8%, Dutch German-Mennonite, 0%). Risk factors for anti-HBc identified from univariate analyses included: being of the Garifuna ethnic group, ($p<.00001$), residence in the Stann Creek district, ($p<.00001$), a reactive syphilis serology, ($p=.02$), a household size of 8 or greater, ($p=.02$), and five or more lifetime sexual partners, ($p=.01$). Health care work, tattoos and intravenous drug use were not identified risks. Multivariate analyses by stepwise logistic regression identified ethnicity, ($p=.0001$), and a reactive rapid plasma reagin (RPR), ($p=.02$), as significant independent predictors of anti-HBc seropositivity. Strategies to screen all pregnant women and provide immunoprophylaxis to susceptible infants may be effective in interrupting neonatal HB in Belize. Highly variable differences in anti-HBc rates by district may permit the targeting of limited public health resources for education and prevention of HB and other sexually transmitted diseases.

Presented at the Commonwealth Caribbean Research Council Meeting for 1994 and ready to be submitted to a journal.

SEROPREVALENCE OF HEPATITIS B MARKERS IN HEALTH-CARE WORKERS IN BELIZE

Shilpa Hakre, BS Linda Reyes, BS, David Cruess, Ph.D, Joe P. Bryan, MD

ABSTRACT

A seroprevalence survey of hepatitis B markers was conducted among health care workers in Belize to help determine the epidemiology of hepatitis B and to determine if screening before immunization might save vaccine costs. Of the 330 tested, 94 (28%) were positive for anti-HBc and 3 (1%) had HBsAg. Anti-HBc increased with age from 12% in those 18-24 years to 52% in those ≥ 50 years. The rate was 17% of 48 men compared with 30% of 282 women ($p=.05$). Rates increased with years of medical service and were higher among nurses (69/228; 30%) and domestic workers (15/44; 34%) than among physicians (0/20). Anti-HBc differed significantly among ethnic groups: Mestizo, 4%; Creole, 33% and Garifuna, 57%. Rates differed by district ranging from 3% in a northern district (mostly Mestizo) to 67% in a southern district (mostly Garifuna). Parenteral exposure to hepatitis B through needle stick injuries and blood transfusions was not associated with anti-HBc. Multiple logistic regression analysis confirmed ethnicity, district of residence and age as the best predictors of anti-HBc. Regional differences in exposure suggest that testing of health care workers for anti-HBc before hepatitis B immunization in the 3 southern districts of Belize may result in vaccine cost savings.

Presented at the 43rd Annual Meeting of The American Society Of Tropical Medicine And Hygiene, Cincinnati, OH; November, 1994.

RANDOMIZED COMPARISON OF 5 AND 10 μ g DOSES OF TWO RECOMBINANT HEPATITIS B VACCINES.

Joe P. Bryan, MD, Peter G. Craig, MD, MTM & H, Philip MacArthy, Ph.D, Linda L. Reyes, BS Shilpa Hakre, BS, Ruth Jaramillo, BS, Llewellyn J. Legters, MD, MPH.

Abstract

The high cost of hepatitis B vaccines remains an obstacle to their use. Since the recommended adult dose of Recombivax HB (MSD) is 10 μ g and that of Engerix B (SKB) is 20 μ g, we sought to determine if 10- μ g doses of each vaccine are equally immunogenic. Further, since 5- μ g doses of Recombivax are routinely used in those \leq 29 years of age in the US military, we sought to compare this dose with 5- μ g doses of Engerix B. Lower doses of Engerix would result in vaccine cost savings.

Methods: Members of the Belize Defence Force who were \geq 18 years of age (median 24) without detectable anti-HBc were randomly assigned to receive Recombivax, 5 or 10 μ g, or Engerix, 5 or 10 μ g IM at 0, 1, & 6 months.

After 3 doses, concentrations of anti-HBs were highest among those receiving Recombivax 10 μ g ($n = 22$) or 5 μ g ($n = 46$) with geometric mean (Geo. mean) anti-HBs of 744 and 570 IU/L, respectively. Similar proportions in the two groups developed \geq 10 IU/L anti-HBs (100% and 98%). Among the 91 persons who received Engerix 10 μ g, the Geo. mean anti-HBs was 325 IU/L and 91% developed \geq 10 IU/L. The 87 persons who received Engerix 5 μ g had the lowest Geo. mean, 177 IU/L ($p < .05$ compared with either Recombivax group). Only 86% attained \geq 10 IU/L anti-HBs ($p > .05$ compared with other regimens). The proportion attaining \geq 100 IU/L was lower in the 5 μ g Engerix group (63%) compared with 80% in the 5- μ g or 95% in the 10 μ g Recombivax groups ($p < .05$). Engerix administered in 5 μ g doses is less immunogenic than 5 or 10 μ g doses of Recombivax. In healthy populations < 30 years of age, regimens of half the recommended adult dose (5- μ g of Recombivax or 10 μ g of Engerix) are highly immunogenic and may result in significant vaccine cost savings.

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VARIATION IN THE HINDTARSAL MARKINGS OF ANOPHELES DARLINGI
(DIPTERA: CULICIDAE) IN BELIZE

Ralph E. Harbach, Ph.D, Donald R. Roberts, Ph.D and
Sylvie Manguin, Ph.D

ABSTRACT

Aberrant phenotypes of *Anopheles darlingi* with basal dark scaling on either one or both of hindtarsomeres 3 and 4 are reported from Belize. Based on wild-caught females and adults reared from wild-caught larvae, it appears that approximately 8% of the natural population bears some degree of basal dark scaling on these hindtarsomeres. The occurrence of similar variants in other species of the subgenus *Nyssorhynchus* is summarized, and their significance in terms of inaccurate species identification is noted.

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**ENVIRONMENTAL AND REGIONAL DETERMINANTS OF *ANOPHELES*
(DIPTERA: CULICIDAE) LARVAL DISTRIBUTION IN BELIZE, CENTRAL
AMERICA.**

Eliska Rejmankova, Ph.D, Donald R. Roberts, Ph.D, Ralph E. Harback, Ph.D, Jim Pecor, EL. Peyton, Sylvie Manguin, Ph.D, Richard E. Krieg, Ph.D, Jorge Polanco, MD, MPH, Llewellyn J. Legters, MD, MPH

ABSTRACT

Surveys of *Anopheles* larval habitats in northern Belize were carried out during September 1990 and April 1991. At each site, larvae were collected and the physical and chemical characteristics of water and species composition of aquatic vegetation were measured or estimated. Data on presence or absence of four species, *Anopheles albimanus* Wiedemann, *A. crucians* Wiedemann, *A. pseudopunctipennis* Theobald, and *A. argyritarsis* Robineau-Desvoidy, were used for analysis of associations with environmental factors, habitat types, and regions. Using significantly contributing environmental variables, discriminant functions (DF) were constructed for the *Anopheles* species, except for *A. argyritarsis* whose distribution could be predicted solely by altitude. The stability of DFs was checked by cross-validation runs. The DF for *A. pseudopunctipennis* was 93% accurate in predicting positive habitats. Predictions based on DFs for *A. albimanus* and *A. crucians* were 74 and 80% accurate, respectively. Of the four *Anopheles* species present in the study area, *A. albimanus* was the most common. Together with *A. crucians*, it occurred mostly on the coastal plain, and both species were present in both wet and dry seasons. *Anopheles albimanus* was positively associated with cyanobacterial mats and submersed-periphyton habitat types and negatively associated with the filamentous algae habitat type. *A. crucians* was positively associated with *Eleocharis*-periphyton habitat type. *A. pseudopunctipennis* and *A. argyritarsis* were common only during the dry season and their distribution was limited to the Karst and Mountain Pine Ridge regions. Both species were positively associated with the filamentous algae habitat type, and *A. argyritarsis* was also positively associated with the rock pools habitat type. Physical factors (e.g., water depth, water temperature, and oxygen content) were usually marginally correlated with larval occurrence. Dominant plant growth forms, such as filamentous algae, cyanobacterial mats, and submersed macrophytes showed the closest association with the larvae of particular *Anopheles* species. Our results demonstrated the controlling influence of dominant aquatic vegetation on larval presence.

Presented at the annual meeting of the Amer. Mosquito Control Association, Corpus Christi, TX March 1992. Also presented at the INTECOL International Wetland Conference, Columbus, Oh September 1992.

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PRELIMINARY OBSERVATIONS ON THE CHANGING ROLES OF MALARIA VECTORS IN SOUTHERN BELIZE

Donald Roberts, Ph.D, O. Chan, Jim Pecor, Eliska Rejmankova, Ph.D, Sylvie Manguin,
Ph.D, Jorge Polanco, MD, MPH and Llewellyn J. Legters, MD, MPH

ABSTRACT

A survey for larval and adult *Anopheles* mosquitoes was conducted in Toledo District of southern Belize during August-September 1992. We surveyed for larvae in 145 habitats and conducted paired indoor-outdoor collections of adult mosquitoes landing on humans at 6 houses. In 1940-41, Kumm and Ram reported *Anopheles darlingi* females to be the most common *Anopheles* mosquitoes inside houses and reported no specimens of *Anopheles vestitipennis* in southern Belize. In our 1992 survey we found no *An. darlingi* mosquitoes either as adults or larvae. More *An. vestitipennis* females were captured indoors than outdoors, whereas most *Anopheles albimanus* and *Anopheles apicimacula* females were captured outdoors. All 3 species were represented occasionally in 145 collections of larvae from diverse habitats. *Anopheles vestitipennis* now appears to be a potentially important vector of malaria during the wet season in Toledo District.

Presented at the Annual Meeting of the American Mosquito Control Association, Sheraton Harbor Place Hotel, Fort Myers, Florida. April 18-22, 1993.

Published in the Journal of the American Mosquito Control Association 9(4): 456-459, 1993.

THE ENVIRONMENT, REMOTE SENSING AND MALARIA CONTROL

Donald R. Roberts, Ph.D., Mario Henry Rodriguez, MD, Ph.D

SUMMARY

Results of studies in California, Mexico and Belize demonstrate the value of remote sensing technology for studying vector-borne diseases. These studies have also shown that it is necessary to fully define the environmental factors associated with the presence of vectors and disease transmission, and to be able to detect these environmental factors with image data. These studies, and other published reports, are demonstrating many potential uses of remotely-sensed data in managing and targeting vector and disease control measures.

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PREDICTIONS OF MALARIA VECTOR DISTRIBUTIONS IN BELIZE USING MULTISPECTRAL SATELLITE DATA

Donald R. Roberts, Ph.D., Jack Paris, Ph.D., Sylvie Manguin, Ph.D., Ralph E. Harbach,
Ph.D., Robert Woodruff, Eliska Rejmankova, Ph.D., Jorge Polanco, MD, MPH, R.
Wullschleger, Llewellyn J. Legters, MD, MPH

ABSTRACT

Use of multispectral satellite data to predict arthropod-borne disease trouble spots is dependent on clear understandings of environmental factors that determine the presence of disease vectors. A blind test of remote sensing-based predictions for the spatial distribution of a malaria vector, *Anopheles pseudopunctipennis*, was conducted as a follow-up to two years of studies on vector-environmental relationships in Belize. Four of eight sites that were predicted to be high probability locations for presence of *An. pseudopunctipennis* were positive and all low probability sites (0/12) were negative. The absence of *An. pseudopunctipennis* at four high probability locations probably reflects the low densities that seem to characterize field populations of this species, i.e., the population densities were below the threshold of our sampling effort. Another important malaria vector, *An. darlingi*, was also present at all high probability sites and absent at all low probability sites. *Anopheles darlingi*, like *An. pseudopunctipennis*, is a riverine species. Prior to these collections at ecologically defined locations, this species was last detected in Belize in 1946.

Invited Paper in the Symposium "Future Predictions and Need" of the First
International Congress of Vector Ecology held in San Diego, CA 3-8 October
1993.

Presented at the joint annual meetings of the American Society of Tropical Medicine and
Hygiene and the American Society of Parasitology held at Atlanta, GA. October 1993.

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1994.

ANOPHELES ALBIMANUS AND CYANOBACTERIAL MATS: AN EXAMPLE OF ACCURATE HABITAT SELECTION

Eliska Rejmankova, Ph.D., Donald R. Roberts, Ph.D., Sylvie Manguin, Ph.D., Kevin Pope, Ph.D., and Rebecca Post, Ph.D.

SUMMARY

1. Extensive herbaceous wetlands occur in northern Belize. those dominated by sparse emergent macrophytes, rushes and sawgrass, often develop floating mats of cyanobacteria (blue-green algae). These mats provide suitable habitat for larvae of *Anopheles albimanus*.

2. Presence/absence of *A. albimanus* larvae and cyanobacterial mats was assessed in marshes located throughout northern Belize. Of the 21 marshes examined during the 1993 wet and 1994 dry seasons, cyanobacterial mats were found in 11, and *A. albimanus* larvae were detected in 9 of these 11 marshes. No *A. albimanus* larvae were found in marshes without CB mats.

3. Mosquito larvae were collected along two 1,000 m long transects in both the wet season (August 1993) and the dry season (March 1994) to delineate larval distribution in marshes with cyanobacterial mats. *Anopheles albimanus* larval densities in CB mats were relatively high in both seasons: 2.8 and 2.3 larvae/dip in the wet and dry seasons, respectively, in Chan Chen marsh; and 0.8 and 1.02 larvae/dip in Buena Vista marsh. Numbers of larvae per dip did not significantly change with increasing distance from houses/pastures or margins of the marsh.

4. A field experiment showed a strong preference of ovipositing *A. albimanus* for cyanobacterial mats. Higher temperatures and higher CO₂ emissions from cyanobacterial mats are possible ovipositional cues.

Submitted for publication in the Bulletin of Entomological Research (1994).

**BIOCHEMICAL SYSTEMATICS AND POPULATION GENETIC STRUCTURE
OF ANOPHELES PSEUDOPUNCTIPENNIS, VECTOR OF MALARIA IN
CENTRAL AND SOUTH AMERICA**

Sylvie Manguin, Ph.D., Donald R. Roberts, Ph.D., EL. Peyton, Idefonso Fernandez-Salas, Ph.D., Mauricio Barretto, Ph.D., Roberto Fernandez Loyaza, Rafael Elgueta Spinola, Renato Martiniez Granaou and Mario H. Rodriguez, M.D., Ph.D.

ABSTRACT

An electrophoretic survey of 42 populations of *Anopheles (An.) pseudopunctipennis* collected throughout its known geographic distribution indicated strong differences in the allele frequencies of three enzyme loci (*GcD*, *6Pgd*, *Pgm*) of the 33 loci analyzed. No fixed electromorphic differences separate the populations of *An. pseudopunctipennis*. The electrophoretic data indicated three clusters, one from North America (USA, Mexico) and Guatemala, one from Belize and South America (Colombia, Ecuador, Peru, Chile, Argentina), and one from the Island of Grenada (type-locality of *An. pseudopunctipennis*). The populations of *An. pseudopunctipennis* showed little genetic divergence with Nei distances ranging from 0 to 0.079. A comparison of *An. pseudopunctipennis* data with either one of three other *Anopheles* species, showed high genetic distance of 0.335 with a closely related species *An. (An.) franciscanus*; 0.997 with *An. (An.) crucians*; and 2.355 with *An. (Nyssorhynchus) albimanus*. Our isozyme analysis showed three geographic populations of *An. pseudopunctipennis*; one from North America (USA, Mexico) and Guatemala; one from Belize and South America (Colombia, Ecuador, Peru, Chile, Argentina); and one from the Caribbean (Island of Grenada). Part of the suture zone between the two mainland geographic populations occurs in the area of western Belize and eastern Guatemala.

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(Submitted), 1994.

CHARACTERIZATION OF *ANOPHELES DARLINGI* (DIPTERA: CULICIDAE) LARVAL HABITATS IN BELIZE, CENTRAL AMERICA

Sylvie Manguin, Ph.D., Donald R. Roberts, Ph.D., Richard Andre, Ph.D., Eliska
Rejmankova, Ph.D. and Shilpa Hakre

ABSTRACT

A survey for larval habitats of *Anopheles darlingi* Root in Central Belize (Cayo and Belize districts) was carried out in April and May, during the dry season, and in August 1994. The results indicated that *An. darlingi* is present throughout the year in different types of bodies of water, such as: (1) river margin (75%), in patches of floating debris (wood debris, flowers, seeds, dead leaves), frequently shaded by bamboo hanging over the banks and dense submersed bamboo roots; (2) lake margin (15%), among submersed roots and patches of floating leaves or dense emergent vegetation (e.g., *Eleocharis* spp. covered with periphyton), from sunlit to partial and heavily shaded areas; (3) small lagoon (5%), in submersed and floating plants (*Cabomba aquatica*), dead leaves and wood debris, partially shaded; (4) ground pool (5%), in submersed and floating plants (*Cabomba aquatica*), dead leaves and wood debris, partially shaded to complete sunlight. Chi-square analysis showed that *An. darlingi* larvae had positive associations with shade ($p < 0.05$) and submersed vegetation ($p < 0.05$), and that *An. albimanus* Wiedemann larvae were strongly associated with presence of algae ($p < 0.05$). Physical protection of *An. darlingi* larvae from the rapid flow of water was provided by submersed tree roots, overhanging branches, and floating logs. In these protected areas, larvae aggregated among the floating debris or floating vegetation. In Belize, *An. albimanus* is an ubiquitous mosquito that oviposits in a wide variety of aquatic habitats, but in contrast, *An. darlingi* is a riverine mosquito that oviposits in highly selected habitats. The association of *An. darlingi* with major river systems in Central America was verified.

Submitted for publication in the Journal of Medical Entomology (1994).

ECOLOGICAL AND EPIDEMIOLOGICAL PARAMETERS OF MALARIA VECTOR DISTRIBUTIONS IN BELIZE

Donald R. Roberts, Ph.D., Sylvie Manguin, Ph.D., Eliska Rejmankova, Ph.D., Shilpa Hakre, Richard Andre, Ph.D., Ralph Harbach, Ph.D., Jorge Polanco, M.D., M.P.H.

ABSTRACT

Human host-seeking behaviors and spatial distributions of adult *Anopheles darlingi* and *An. albimanus* populations were studied in the central and northern regions of Belize. Data were derived from paired indoor and outdoor collections of mosquitoes landing on humans from 6:30 to 8:00 p.m. Landing captures were conducted at a total of 26 houses in riverine sites in central Belize during the dry and wet seasons of 1993 and the dry season of 1994; and at 16 houses in villages on the northern coastal plain during the dry season of 1994. *Anopheles darlingi* populations were relatively common along the Sibun River from the Hummingbird Highway to La Democracia. The limits of *An. darlingi* distribution along this river in feet above sea level or proximity to the ocean are undefined. Females of *An. darlingi* were found to be more endophagic and, consequently, more likely to bite humans than *An. albimanus* females. A synthesis of numerical abundance and spatial distribution data from riverine areas shows that *An. darlingi* is the dominant indoor-biting *Anopheles* mosquito and, as a consequence, is probably the major vector of malaria along the Sibun River and other river systems, including rivers, streams and creeks in Belize. Alternatively, in northern coastal lowland areas of Belize, *An. albimanus* is greatly abundant in wetland-associated villages where it probably serves as the primary vector of human malaria.

Presented at the Annual meeting of the American Society of Tropical Medicine and Hygiene, Nov. 17, 1994 at Cincinnati, OH.
Prepared for submission for publication in the Journal of Medical Entomology, 1994.

PREDICTIONS OF ADULT *ANOPHELES ALBIMANUS* DENSITIES IN VILLAGES BASED ON DISTANCES TO REMOTELY SENSED LARVAL HABITATS

Eliska Rejmankova, Ph.D., Donald Roberts, Ph.D., Anitra Pawley, Sylvie Manguin,
Ph.D. and Jorge Polanco, M.D., M.P.H.

ABSTRACT

Remote sensing is particularly helpful for assessing the location and extent of vegetation formations, such as herbaceous wetlands that are difficult to examine on the ground. Marshes that are sparsely populated with emergent macrophytes and dense cyanobacterial mats have previously been identified as very productive *Anopheles albimanus* larval habitats. This type of habitat was detectable on a classified RS image of northern Belize (SPOT multispectral XS) as a mixture of two isoclasses. A similar spectral signature is characteristic for vegetation of river margins consisting of aquatic grasses and water hyacinth, that constitutes another productive larval habitat. Based on the distance between human settlements (sites) of various sizes and the nearest marsh/river exhibiting this particular class combination, we selected two groups of sites: those located closer than 500 m and those located more than 1,500 m from such habitats. Prediction accuracy was tested by conducting paired indoor-outdoor landing collections of mosquitoes at one house per site. A simple mathematical model showed that a biting rate of 0.5 bites/man/min. from 18:30 to 20:00 h was the threshold for epidemiologically important high (>0.5 bites/human/min.) versus low (<0.5 bites/human/min.) densities of *A. albimanus* mosquitoes. Houses located less than 500 m from the habitat were predicted as having values higher than this threshold, while lower values were predicted for houses located >1,500 m from optimal habitats. Predictions were verified by collections of mosquitoes landing on humans. The predictions were 100% accurate for houses in the >1,500 m category and 89% accurate for houses in the <500 m category.

Presented at the Annual meeting of the Amer. Soc. Trop. Med. Hyg, Nov. 17, 1994 at Cincinnati, OH

Prepared for submission for publication in the American Journal of Tropical Medicine and Hygiene, 1994.

SEROPREVALENCE OF HEPATITIS B AMONG SCHOOL AGED CHILDREN IN STANN CREEK DISTRICT, BELIZE

Judith Chamberlin, PAC, Joe P. Bryan, MD, David L. Jones, MD, Linda Reyes, BS,
Shilpa Hakre

Adults in Stann Creek District, Belize have a high prevalence of hepatitis B virus (HBV) infection, but the age at onset of these infections is unclear. We conducted a seroprevalence study of hepatitis B markers among school-aged children in that district to provide information for planning a hepatitis B vaccine program. After informed parental consent, 587 students, aged 4-22 (mean 13 years), from five schools were tested for antibody to hepatitis B core antigen (anti-HBc) and hepatitis B surface antigen (HBsAg). The overall prevalence of hepatitis markers was high: 43.3% had anti-HBc and 7.7% had HBsAg. Anti-HBc was more common in males than females (52% vs. 36.5%; $p < .05$). There was also marked variation between ethnic groups.

	Mayan (n=133)	Mestizo (n=92)	Garifuna (n=173)	Creole (n=141)	Other (n=48)
Anti-HBc +	76%	50%	37%	25%	19%
HBsAg +	9%	11%	9%	4%	2%

At the two rural primary schools attended mainly by Mayan and Mestizo children, >65% of children ≤ 8 years had anti-HBc, with no increase with age. In contrast, at the urban primary school attended mainly by Garifuna and Creole children, only 9% of children ≤ 8 years had anti-HBc, and seropositivity increased with age ($p < .05$, by chi-square test for trend). Anti-HBc was found in 42% and 36% of students at the two high schools. In conclusion, among school-aged children in Stann Creek District, the prevalence of HBV infection varies by location and ethnicity. Because most children in the rural areas, are exposed to hepatitis B before entering school, immunization against HBV should be integrated into the routine infant immunization program.

Submitted for consideration for presentation at the Annual Meeting of the American Society for Tropical Medicine and Hygiene in San Antonio, November, 1995

HEPATITIS E VIRUS EXCRETION AND SEROLOGIC RESPONSE FROM AN EPIDEMIC OF HEPATITIS IN PAKISTAN

Hua Yuan Zhang, Muhammad Iqbal, Joe P. Bryan, Sergei Tsarev, Charles F. Longer, Abdul Rauf Rafiqui, J. Fred Duncan, Jeffrey D. Caudill, Aftab Ahmed, Asad Khan, Ahmed Khan, Thomas A. Miele, Iftikhar A. Malik, Robert H. Purcell, John Ticehurst, and Llewellyn J. Legters

Hepatitis E virus (HEV) is an important cause of sporadic and epidemic hepatitis in Southwest Asia. An outbreak of hepatitis which occurred among men in an academic community in Abbottabad, Pakistan, was traced to contamination of a water supply. Of 109 men hospitalized with a clinical diagnosis of hepatitis, 104 (95%) were found to have serologic evidence of acute hepatitis E. Both IgM and IgG anti-HEV were present in 92% of cases on admission. Among 44 men from whom three serum specimens were obtained over 4 months, IgG anti-HEV geometric mean titers (GMT) decreased from 1534 on admission to 651 at 4 months. IgM anti-HEV was detected in 40 (91%) of 44 men at a GMT of 525 during acute disease but was observed in only 8 (18%) four months later. Affinity-capture PCR (AC/PCR) detected HEV in serially-collected feces from 18 of 19 men. The intensity of viral excretion was greatest during the first week of symptoms (present in 79% of patients) but persisted intermittently for up to 4 weeks after the onset of jaundice. PCR studies of single fecal specimens from 41 patients detected HEV in 13 (32%). This study identifies HEV as the etiologic agent of this outbreak, elucidates the pattern of anti-HEV in patients, and demonstrates the pattern of HEV excretion in feces as measured by AC/PCR.

Submitted for consideration for presentation at the Annual Meeting of the American Society for Tropical Medicine and Hygiene in San Antonio, November, 1995

the amount of CS protein released were dependent upon incubation times and temperatures. There were no differences in the amount of CS protein released by sporozoites from either the salivary glands, the hemolymph or mature oocysts, or those obtained from mosquitoes 16 or 25 days post-infection. Contact with immune sera in the mosquito host did not affect the ability of sporozoites to release CS protein. The amount of CS protein released during incubation of sporozoites from 45 individual mosquitoes was almost half of the amount of free CS protein within the salivary glands. The amount of CS protein per sporozoite, either released during incubation or free in the glands, was inversely related to the numbers of sporozoites per sample. Further experiments indicated that sporozoites regulate their production of CS protein according to background levels of soluble CS protein. Such mechanisms in the mosquito host may contribute to the viability and transmission potential of sporozoites.

- 133 THE QUANTITATIVE BUFFY COAT SYSTEM (QBC) FOR THE RAPID DIAGNOSIS OF *PLASMODIUM FALCIPARUM*, *P. VIVAX* AND *P. MALARIAE* IN A HYPERENDEMIC COMMUNITY. Anthony RL*, Purnomo, and Bangs MJ. US Naval Medical Research Unit, No. 2, Jakarta, Indonesia; and The Department of Pathology, University of Maryland School of Medicine, Baltimore, MD.

The QBC malaria diagnostic system was used for the detection and identification of malaria parasites in blood specimens collected from 322 residents of Oksibil; an isolated highland valley in the eastern Jayawijaya Mountains of Irian Jaya, Indonesia. The availability of a rechargeable centrifuge and a fiber-optic paralens, which permits visualization of the plasmodia without the need of a fluorescent microscope, enabled us to complete and interpret the assay in this remote environment. Parasites were easily identified in 53 of 59 specimens which were subsequently confirmed as positive on matched Giemsa stained thick smears (sensitivity = 89.8%). Of the 6 specimens with disparate results, 3 were from cases of *P. falciparum* showing gametocytes only, 1 was from a light *P. ovale* infection and 2 were identified as *P. vivax*. Twenty-three of 263 specimens which were negative by thick smear were positive by QBC (specificity = 91.2%). Fourteen of these specimens were interpreted as "rare *P. vivax* rings", 2 were regarded, and later reconfirmed, as *P. malariae* and 7 were identified as *P. falciparum*. Of the 53 plasmodia identified by thin smear, 26 were identified correctly by QBC; 20/24 *P. falciparum*, 4/22 *xP. vivax* and 2/7 *P. malariae*. Most of the 27 discordant results were attributed to lack of experience in distinguishing between light infections of *P. vivax* and *P. falciparum*. In spite of these difficulties, it was concluded that the QBC is an easy, sensitive and rapid method for diagnosis of malaria in the field and that it provides an additional means for the identification of those individuals who are in need of treatment.

POSTER I: ARBOVIRUS AND HEPATITIS

- 134 HEPATITIS IN NORTHERN PAKISTAN. Bryan JP*, Rauf A, Ahmed A, Perine PL, Malik IA, and Legters LJ. Department of Preventive Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD; and Pakistan-U.S. Laboratory for Sero-Epidemiology (PULSE), Rawalpindi, Pakistan.

The etiology of hepatitis in 1129 men admitted to Military Hospital (MH) in Rawalpindi, Pakistan between February 1987 and November 1990 was studied. During this period, hepatitis B was the etiology in 311 (28%) while 804 (71%) were non-A, non-B cases. Hepatitis A was the etiology in only 13 (1%) cases. However, IgG anti-HAV was present in all 179 sera tested, indicating prior hepatitis A infection. Over these 4 years, the proportion of cases of NANB increased from 65% to 77% per year ($p = 0.006$) and the proportion of cases of acute hepatitis B decreased from 32% to 23% $p = 0.003$. Between 1987-89, one of 70 patients with HBsAg was positive for anti-delta antibody. Between 1987-89, HBsAg was present in 169 (26%) of 646 cases of NANB hepatitis while in 1990, at least 14% of NANB cases had HBsAg indicating convalescent or chronic carriage. The mean age of patients during these four years remained constant at 31 years. Patients with HBV or NANB were of similar age. The total bilirubin was

significantly higher in patients with NANB to hepatitis B (10.4 \pm 0.8 vs 8.3 \pm 0.6) as compared to patients with NANB to hepatitis A (4.2%; $p = 0.012$), to have hepatitis E (7.9%; $p < 0.001$). Infection with hepatitis E has been a major cause of non-A, non-B hepatitis.

- 135 RIFT VALLEY FEVER (RVF) IN ANTANANARIVO, MADAGASCAR. Morvan J, Rollin PE, and Ratsimbazafy A.

Between February and April 1977, sixteen aborted fetuses and one stillborn infant were isolated from human laboratory in Antananarivo during the 1977 epidemic. The isolates were done by IFA, IgM capture ELISA, and RVI. All were negative or very low RVI. Between 40 and 91% of the isolates were positive for IFA and IgM ELISA. One of 11,597 mosquitoes (58%) was positive for RVF. Extensive studies are being conducted on the high

- 136 TITERS OF VESICULOPATHIC VIRUS (VPU) IN SHANNON ISLAND, ILLINOIS. Stalknecht DE, Corbett RE, and College of Veterinary Medicine.

Vesicular stomatitis virus (VSV) was isolated from Shannon Island, Georgia, in 1990, 7973 *L. shannoni* were isolated from Vero cells. Four isolates were identified as VSV. One isolate was from a pool of damaged units of virus per replication. This study provides evidence for transovarial transmission of VSV.

- 137 NATURAL GENETIC VARIATION IN THE IDENTIFICATION OF ARBOVIRUS RESEARCH.

Primer-extension sequencing of Japanese encephalitis (JE) viruses has been used to study the evolution. In a previous study

significantly higher in patients with acute hepatitis B on clinical presentation than non-A, non-B hepatitis (10.4 ± 0.8 vs 8.3 ± 0.6) as was the ALT (480 ± 65 vs 263 ± 16). Patients with HBV were more likely than patients with NANB to have had domiciliary or sexual contact with a patient with hepatitis (12.4% vs 4.2%; $p = 0.012$), to have received injections (39% vs 30%; $p=0.12$) or to have had dental treatment (26% vs 7.9%; $p<0.001$). Infections with hepatitis A, B and agents of NANB are common in the Pakistan military. Since hepatitis E has been documented in other military settings and in travelers to Pakistan, it may be a major cause of non-A, non-B hepatitis.

135 RIFT VALLEY FEVER EPIZOOTIC IN THE CENTRAL HIGHLANDS OF MADAGASCAR.

Morvan J, Rollin PE*, Laventure S, Rakotoarivony I, Coudrier D, and Roux J. Institut Pasteur de Madagascar, Antananarivo, Madagascar; and Institut Pasteur, Paris, France.

Between February and April 1991, Official Veterinary Services reported an unusual number of bovine abortions around Antananarivo (central highlands, Madagascar). RVF virus isolations were made from sixteen aborted fetus and one dead calf in different foci. By monoclonal antibody characterization, the isolated viruses were found identical to the 1979 RVF strains isolated in Madagascar from mosquitoes and human laboratory infection. These strains were more related to the Egyptian RVF strains isolated during the 1977 epidemic than from any other African strains. Serological surveys in bovine and human were done by IFA, IgM capture ELISA and all positive sera were confirmed by PRNT. In the formerly negative or very low RVF antibody prevalence bovine population, we found a high prevalence of IgM antibodies in bovine (267/968, 27.5% positives). The IgM prevalence in recently aborting females varied between 40 and 91%. Among 490 human sera tested, respectively 10.4 and 6.1% were found positive by IFA and IgM ELISA. One RVF human death was confirmed by virus isolation and specific IgM. A total of 11,597 mosquitos (58% *Culex antennatus*) were collected in the epizootic areas and tested. No isolation was made. Extensive studies were conducted to determine the geographical extension and the impact of this epidemic on the highly susceptible livestock and human populations.

136 TITERS OF VESICULAR STOMATITIS VIRUS, NEW JERSEY SEROTYPE, IN MALE AND FEMALE *LUTZOMYIA SHANNONI* (DIPTERA: PSYCHODIDAE) COLLECTED IN GEORGIA. Comer JA*, Stallknecht DE, Corn JL, and Nettles VF. Southeastern Cooperative Wildlife Disease Study, College of Veterinary Medicine, The University of Georgia, Athens, GA.

Vesicular stomatitis viruses are important pathogens of cattle, horses, and swine in the American tropics and the United States. The New Jersey serotype of vesicular stomatitis virus (VSNJ) is enzootic on Ossabaw Island, Georgia, and *Lutzomyia shannoni*, a phlebotomine sand fly, is the suspected vector. In 1990, 7973 *L. shannoni* were collected from hollow trees on the island and processed for virus isolation in Vero cells. Four isolates of VSNJ were obtained: two from pools of female, one from a pool of male, and one from a pool of damaged, unsexed sand flies. Three pools contained between $10^{4.3}$ and 10^5 plaque-forming units of virus per pool, suggesting that the positive flies within these pools had supported VSNJ replication. This study provides further evidence that *L. shannoni* is a biological vector of VSNJ and that transovarial transmission of the virus occurs in nature.

137 NATURAL GENETIC VARIATION AMONG JAPANESE ENCEPHALITIS VIRUS STRAINS; IDENTIFICATION OF A NEW GENOTYPIC GROUP. Chen WR*, Rico-Hesse R, and Tesh RB. Yale Arbovirus Research Unit, Yale University School of Medicine, New Haven, CT.

Primer-extension sequencing of the RNA templates of polio, dengue, Rift Valley fever and Japanese encephalitis (JE) viruses has provided new information on their geographic distribution, origin and evolution. In a previous study of 46 diverse JE virus strains, we demonstrated the existence of 3 distinct

**INTERNATIONAL SYMPOSIUM ON
VIRAL HEPATITIS AND LIVER DISEASE**
(*The 8th Triennial Congress*)

***SCIENTIFIC PROGRAM
AND ABSTRACT VOLUME***

**May 10-14, 1993
Tokyo**



VIRAL HEPATITIS RESEARCH FOUNDATION OF JAPAN (VHFJ)



ABSTRACT*

ANTIBODY TO HEPATITIS E VIRUS (ANTI-HEV) IN SERIAL SERA FROM AN OUTBREAK: DETECTION BY ENZYME IMMUNOASSAY (EIA).

J Ticehurst, PO Yarbough, JP Bryan, E Garza, JD Caudill, AR Rafiqui, A Ahmed, M Iqbal, IA Malik, JF Duncan, LJ Legters. Walter Reed Army Inst Res, Washington DC, Genelabs Diagnostics, San Antonio TX, Uniformed Services Univ Health Sciences, Bethesda MD, USA, Army Medical College, Rawalpindi, Pakistan.

Objectives/methods: A hepatitis outbreak during August 1988 resulted from fecal contamination of the water supply for a housing unit. To determine patterns of antibody reactivity, EIA (Table) was used to test 27% of the sampled individuals.

Results: Anti-HEV IgM was detected in 43% of Cases during Aug; IgG, in 97%. By Sept, "seroconversions" occurred: IgM developed in 5 Cases; IgM and IgG in 1 Normal; IgG in 1 Case and 2 Contacts. Conversely, IgM was no longer detected in 2 Cases; IgG was not detected in 1 Normal who had IgG in Aug and Dec. By Dec, IgM was no longer detected in 100% of Cases; 2 Cases (11%) and 1 Contact no longer had IgG. Case #7 had IgG and IgM in Aug but neither in Dec; by immune electron microscopy (IEM), both sera had high levels of anti-HEV.

Discussion: Anti-HEV IgM was detected in 61% of Cases within 1 month of jaundice and in 0% by 4 months. Thus, EIA readily identified HEV as the probable cause of the outbreak; it was also shown [H-Y Zhang et al, submitted for this symposium] that Cases, including an IgG+/IgM- patient, excreted HEV. We did not determine if other IgG+/IgM-results indicated current or past infection, or nonspecificity. In general, however, IgG was most frequently detected in Cases. Development of anti-HEV among non-cases suggested that anicteric infections occurred. It appeared that anti-HEV IgG sometimes waned, consistent with earlier results. However, the significance of the EIA-IEM discrepancy for Case #7 is not known.

Table. EIA^a: detection of anti-HEV in sera from Abbottabad, Pakistan^b

	Cases	IgM	IgG	Contacts	IgM	IgG	Normals	IgM	IgG
Aug		11/26 ^c	29/30		0/10	2/10		0/16	3/16
Sept		8/18	17/17		0/7	3/7		1/10	2/10
Dec		0/19	17/19		0/3	2/3		0/1	1/1

^a R Goldsmith et al, Lancet 339:328-331, 1992

^c No. positive / no. assayed

^b Collected in Aug, Sept, and Dec 1988 (most individuals contributed 2 or 3 sera) from Cases (jaundiced patients), Contacts (ill but not icteric), and Normal persons

* An error in the table (fraction for Cases/IgM/Aug) has been corrected

ABSTRACT[§]

PATTERNS OF HEPATITIS E VIRUS (HEV) EXCRETION FROM SELECTED PATIENTS IN AN OUTBREAK. HY Zhang, JD Caudill, TJ Popkin, JF Duncan, I Hussain, A Ahmed, M Iqbal, IA Malik, LJ Legters, J Ticehurst. Walter Reed Army Inst Research, Washington DC, National Insts Health & Uniformed Services Univ Health Sci, Bethesda MD, USA, Army Medical College, Rawalpindi, Pakistan.

Objectives/methods: An earlier study determined, by immune electron microscopy (IEM) of single specimens, that HEV was predominantly excreted during the 1st week of jaundice [J Med Virol 36:84-92, 1992]. During a 1988 outbreak, multiple feces were collected from 19 patients. To increase the likelihood of detecting HEV and to determine excretion patterns, early acute-phase collections were assayed by affinity-capture/polymerase chain reaction (AC/PCR). **Results:** HEV was detected 3-16 days into icterus, but only 29% of specimens were AC/PCR-positive (Table). HEV excretion was intermittent from 2 patients. HEV was detected in 4 of 10 specimens diluted 10^4 . 1 of 2 samples had an AC/PCR titer of 10^6 . **Discussion:** HEV excretion was detected from 5 of 11 patients and during the 3rd week of illness. However, HEV concentrations were usually very low, at or below IEM-detectable levels. Such data were consistent with earlier IEM and PCR results. Case #13 was anti-HEV IgM-negative [Ticehurst et al, submitted for this symposium]; thus, AC/PCR could be used for diagnostic support when suspected cases are anti-HEV IgM-negative.

Table. AC/PCR*: detection of HEV in feces from Abbottabad, Pakistan

Case #	Days after onset of scleral icterus																
	2	3	4	5	6	7	8	9	10	11	12	13	16	19	22	25	
1				-	-												
4				-													
7					-												
9					+	-	-										
11					-												
13					+	+											
63			-														
64			-	+	-	-	-				-						
65				-	-	-	-										
67	-	+	-		-							-	+				
68	-	+	+	+			-		+			-	+	-	-	-	

Certain specimens from this group were assayed by IEM: HEV particles were not detected

* TA Miele et al, abstract submitted for this symposium

[§] Errors have been corrected in the table (Cases 7, 9, 11, 13, 63)



DEPARTMENT OF THE ARMY

U.S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES
FORT DETRICK, FREDERICK, MARYLAND 21702-5011



REPLY TO
ATTENTION OF:

December 21, 1992

Medical Division

Dr. Joe P. Bryan
Department of Preventive Medicine
Uniformed Services University
of the Health Sciences
F. Edward Hebert School of Medicine
4301 Jones Bridge Road
Bethesda, MD 20814-4799

Dear Dr. *Joe* Bryan:

Thank you for the reference copy of the letter sent to Mr. Aftab Ahmed at the PULSE, Rawalpindi, Pakistan.

In reading the letter, I came across your statement that I had worked with samples from Mardan and Quetta. Indeed I did not have the opportunity to work with those samples. The samples I worked with were collected from seven patients admitted to the Military Hospital in Rawalpindi. The collection was made by Dr. Innis, Dr. Redfield, and Dr. Perine in the summer of 1985. An abstract was submitted to the Japan International Hepatitis Symposium, and a manuscript is in preparation. Dr. Legters has agreed with this plan and is communicating with co-authors in Pakistan. For your information a copy of the abstract is enclosed.

Sincerely,

Maria

Maria H. Sjogren, M.D.
Lieutenant Colonel, U.S. Army
Chief, Medical Division

Enclosure

Copy furnished:
Dr. Legters
Dr. Ticehurst

ASSESSMENT OF SEROLOGICAL RESPONSES DURING ACUTE AND CONVALESCENT STAGES OF HEPATITIS E VIRUS INFECTION USING A NEW IMMUNOASSAY. M.H. Sjogren, B. Innis, R. Redfield, P. Perine, J. Ticehurst, I. Mushahwar, G. Dawson, I. Malik, A. Ahmed, F. Duncan, L. Legters, R.H. Purcell. Walter Reed Army Institute of Research, Washington, DC; Abbott Laboratories, Chicago, IL; Uniformed Services University of the Health Sciences, Bethesda, MD; Pakistan-United States Laboratory for Seroepidemiology, Rawalpindi, Pakistan; National Institutes of Health, Bethesda, MD.

Hepatitis E virus (HEV) infection occurs in several areas of the world. An immunoassay would expedite HEV diagnosis and help us understand its epidemiology. We evaluated 7 Pakistani patients whose sera and stool specimens were collected during acute and convalescent stages of "non-A, non-B, non-C hepatitis." Serum from one of these patients had antibody to HEV shown by immune electron microscopy. Four patients had liver biopsies compatible with diagnosis of acute HEV hepatitis. Mean alanine aminotransferase (ALT) was 691 IU/L, and mean bilirubin was 6 mg/dL during the acute phase. All 7 patients recovered uneventfully and had normal ALT and bilirubin 6 months later. Stool suspensions from these patients successfully transmitted HEV to 9/14 inoculated chimpanzees or marmosets. Recently, immunoassays to detect antibodies to HEV have been reported (Dawson et al, J. Virol. Methods, 38, 175, 1992). The aim of our study was to use these assays to confirm HEV infection in these patients. In addition, we tested 28 control sera; 18/28 sera were from Pakistani patients diagnosed as having other (non-HEV) acute viral hepatitis. Results: All acute HEV samples (within 7 days of onset of symptoms) and samples collected 1 month after diagnosis had detectable IgG and IgM anti-HEV. Convalescent samples (6 months after onset) showed persistence of IgG in 5/6 tested; only one sample had IgM anti-HEV during late convalescence. All 28 control samples were negative for IgG anti-HEV. IgM anti-HEV was detected in 3/18 Pakistani hepatitis controls and in none of the 20 control sera. It is unclear whether these IgM-positive sera represent a false positive result or co-infection with another virus. In conclusion, the Abbott immunoassays were useful in identifying HEV-infected patients. In addition, the disappearance of IgM anti-HEV during convalescence and the persistence of IgG anti-HEV were consistent with the clinical evolution and resolution of acute disease. Diagnosis and epidemiology of HEV infection will be greatly facilitated by these assays.

Prevalence of IgG Antibody to Specific Viruses in Four Belizean Populations

VIRUSES¹

Population #	LEPT	DEN2	SLE	FLAV ²	VEE	MAY	EEE	WEE	ALPHA ³	VSVI	VSVN	CGL	PTG	CHG	HTN	P360	FTS
Cowpen 392	18.6	12.5	21.7	3.1	36.5	0.25	1.3	0.25	13.5	18.6	51.3	24.2	3.3	1.3	4.3	1.0	6.6
Caracol 45	22.2	24.4	8.9	4.4	22.2	-	8.9	-	2.2	-	-	-	-	0	2.2	2.2	6.7
Malaria 42	19.0	2.4	26.2	2.4	23.8	0	0	0	0	4.8	9.5	19.0	-	-	4.8	2.4	16.7
FUOS 61	27.9	3.3	62.3	4.9	26.2	1.7	1.6	0	3.3	3.4	8.5	-	1.7	18.0	3.3	0	32.8

¹Key:

LEPT -- Leptospirosis
DEN2 -- Dengue 2
SLE -- St. Louis encephalitis
FLAV -- Indeterminate flavivirus
VEE -- Venezuelan equine encephalitis
MAY -- Mayaro
EEE -- Eastern equine encephalitis
WEE -- Western equine encephalitis

ALPHA -- Intermediate alphavirus
VSVI -- Vesicular stomatitis virus, Indiana strain
VSVN -- Vesicular stomatitis virus, New Jersey strain
CGL -- Changrinola
PTG -- Punta Toro
CHG -- Chagres
HTN -- Hantaan
P360 -- Puumala-like hantavirus

FTS -- Fort Sherman

²Indeterminate; approximately equal ODs to DEN2 and SLE at dilution tested

³Indeterminate; approximately equal ODs with 2 or more alphavirus (VEE, MAY, EEE, WEE), most usually VEE and EEE, at dilution tested.

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- WORLD AND REGIONAL SCENARIO**
- BELIZEAN SCENE :GENERAL BACKGROUND**
- OVERVIEW HIV/AIDS DATA OF BELIZE**

PART B:

DEVELOPING HIV SENTINEL SURVEILLANCE

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Addendum to Final Report

Publications, Masters in Public Health or Tropical Medicine and Hygiene and Doctor of Philosophy Theses from Belize and Pakistan

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Randomized comparison of 5 and 10 μg doses of two recombinant hepatitis B vaccines

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The high cost of hepatitis B vaccines remains an obstacle to their use. Since the recommended adult dose of Recombivax HB (MSD) is 10 μg and that of Engerix B (SKB) is 20 μg , we sought to determine if 10 μg doses of each vaccine are equally immunogenic. Further, since 5 μg doses of Recombivax are routinely used in those ≤ 29 years of age in the US military, we sought to compare this dose with 5 μg doses of Engerix B. Lower doses of Engerix would result in vaccine cost savings.

Methods: members of the Belize Defence Force who were ≥ 18 years of age (median 24) without detectable anti-HBc were randomly assigned to receive Recombivax, 5 or 10 μg , or Engerix, 5 or 10 μg IM at 0, 1, and 6 months. Randomization was weighted toward Engerix.

Results: after 3 doses, geometric mean concentrations (GMC) of anti-HBs were highest among those receiving Recombivax 10 μg ($n=22$) or 5 μg ($n=46$) with GMC anti-HBs of 744 and 570 mIU ml^{-1} , respectively. Similar proportions in the two groups developed $\geq 10 \text{ mIU ml}^{-1}$ anti-HBs (100 and 98%). Among the 91 people who received Engerix 10 μg , the GMC anti-HBs was 325 mIU ml^{-1} and 91% developed $\geq 10 \text{ mIU ml}^{-1}$. The 87 people who received Engerix 5 μg had the lowest GMC, 177 mIU ml^{-1} ($p < 0.05$ compared with either Recombivax group). Only 86% attained $\geq 10 \text{ mIU ml}^{-1}$ anti-HBs ($p > 0.05$ compared with other regimens). The proportion attaining $\geq 100 \text{ mIU ml}^{-1}$ was lower in the 5 μg Engerix group ($p < 0.05$).

Conclusions: Engerix administered in 5 μg doses is less immunogenic than 5 or 10 μg doses of Recombivax. In healthy populations < 30 years of age, regimens of half the recommended adult dose (5 μg of Recombivax or 10 μg of Engerix) are highly immunogenic and may result in significant vaccine cost savings.

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NO. 1 Hepatitis B, Hepatitis B Vaccine, Belize, Central America

Hepatitis B vaccines are very expensive when used in recommended doses in adults in developed countries. The federal contract price in the United States for the approved adult dose of Recombivax HB (Merck Sharp and Dohme) which was licensed in 1986 remains at \$92. One method of saving limited vaccine funds has been to use lower doses. By decreasing the dose of Recombivax from 10 μg to 2.5 μg for children, the cost for the three-dose series is now similar to other childhood vaccines such as the triple vaccines for measles-mumps-rubella or diphtheria-pertussis-tetanus. Several studies

have shown that Recombivax HB given at 5 μg in 0.5 ml, the approved dose recommended for those 11–19 years of age, is immunogenic in those ≤ 29 years of age^{1–3}. The US military has been using 5 μg doses in this age group for several years⁴. The extension of this 5- μg regimen by one decade results in a cost reduction to \$46 per person.

Engerix B (SmithKline Beecham), a recombinant hepatitis B vaccine licensed in the United States in 1989, is recommended at an adult dose of 20 μg . If 5- μg doses of Engerix B were equally immunogenic compared with 5- μg doses of Recombivax HB, then its use could decrease the cost of immunizing adults to one-fourth of the present cost of immunization with Engerix and to one-half the cost of immunization with 5- μg doses of Recombivax. The objective of our study was to compare the immunogenicity and reactogenicity of these two recombinant hepatitis B vaccines in doses of 5 and 10 μg to determine the cost-saving potential of reduced doses.

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Table 1 Characteristics of evaluable volunteers

	Enerix 5 μ g	Enerix 10 μ g	Recombivax 5 μ g	Recombivax 10 μ g
No. of volunteers	94	100	49	25
Males	91	97	46	24
Mean age	24.9	24.8	25.4	26
Age range	18-42	17-39	18-37	18-38
No. (%) ≥ 30 years	17 (18%)	18 (18%)	12 (24%)	7 (29%)
Mean weight (lb)	159	157	156	158
Weight range	110-269	114-224	117-246	118-215
Mean body mass index	25.2	24.7	25.4	25.3
Body mass index >27.7	18 (19%)	16 (16%)	10 (20%)	5 (20%)

METHODS

Human subjects

After approval of the protocol by the Human Use Review Committees of the Ministry of Health in Belize and the Uniformed Services University of the Health Sciences, healthy, active-duty members of the Belize Defence Force who had previously tested negative for anti-HBc⁵ were invited to participate. After signing the informed consent document, volunteers were randomized to one of four vaccine groups.

1. Enerix B, 5 μ g in 0.25 ml
2. Enerix B, 10 μ g in 0.5 ml
3. Recombivax HB, 5 μ g in 0.5 ml
4. Recombivax HB, 10 μ g in 1.0 ml

Randomization was weighted toward the Enerix groups in a ratio of 4:4:2:1. Weighting was done because only \$5000 were available for the purchase of the vaccines. The cost of vaccine per person in each of the four groups was \$6.75, \$11.50, \$46, and \$92, respectively. The Enerix was purchased for use in Belize for \$9.00/20 μ g in 1 ml while Recombivax was purchased in the United States at \$92/30 μ g in 3 ml.

All doses were administered IM in the deltoid muscle at months 0, 1 and 6 using a 5/8-inch, 27-gauge, U-100 insulin syringe (Becton Dickinson). This type of syringe ensured accurate measurement of the vaccine volume and ensured that all vaccine was injected because the needle has no hub. The vaccine recipients were generally lean and muscular, thus ensuring intramuscular injection even with a 5/8-inch needle.

Blood collections of 7 ml were performed 2, 6, and 7 months after the first dose.

Laboratory examinations

Initial screening had been conducted using reference 5 Hepanostika Anti-core (Organon-Teknika Corporation) on sera collected between 24 June and 23 October 1992⁵. Immunization was begun between 22 September and 26 November 1992, the final serum from each vaccine volunteer was tested for anti-HBc using Corzyme (Abbott) to rule out intercurrent infections. Subjects with reactive results by Corzyme were excluded from analysis.

Sera were assayed for anti-HBs using the AUSAB-EIA. Quantitation of anti-HBs was accomplished by comparison with known standards (AUSAB Quantitation Panel, Abbott Labs). The lower limit of detectability was 0.1 mIU ml⁻¹.

Statistics

Proportions were compared using chi-square with Yates correction when appropriate. For purposes of calculating geometric mean concentrations (GMC) and *t*-tests, specimens with no detectable anti-HBs were arbitrarily assigned a value of 0.09 mIU ml⁻¹, since the lower limit of detectability was 0.1 mIU ml⁻¹. Differences between group means were compared by non-paired two-tailed *t*-tests on log-transformed values using Microsoft Excel 4.0 (Microsoft Corp., Redmond, Washington) or Statview 2 (Brainpower Inc., Calabasas, CA). Post study calculations of power (the probability of detecting a significant difference when a difference really exists) were performed⁶.

RESULTS

Three hundred and one people entered the trial and received at least one dose of vaccine. The self-reported ethnicity of these volunteers was Creole (35%), Garifuna (22%), Mestizo (26%), Mopan or Ketchi Mayan (15%) and East Indian (2%). Seven subjects left the Belize Defense Force before any post-immunization serum was collected. Twenty-six others were eventually found to have anti-HBc and were not analyzed. These were almost exclusively among persons of Creole or Garifuna ethnicity, groups that had prevalence rates of anti-HBc of 30 and 56%, respectively, in the screening phase⁵. After randomization, the 268 remaining people were evenly matched in terms of gender, mean age, the proportion of volunteers who were at least 30 years of age, and weight (Table 1).

Reports of local pain were received from 263 (98%) of vaccinees after dose 1. Significantly more (46%) of vaccinees who received Recombivax HB, 10 μ g in 1.0 ml reported pain compared with 11-23% in the other groups ($p < 0.05$). Two people reported severe pain after dose 1; one in each of the 10- μ g groups. After dose 2, similar results were obtained: among those receiving Recombivax 10 μ g, pain was reported in 55% compared with 8-18% in the other groups.

One month and 6 months after beginning immunization, the proportion of persons with anti-HBs ≥ 10 mIU ml⁻¹ was significantly higher in each group receiving Recombivax than in either group receiving Enerix B (Table 2).

One month after the third dose, 86% of those who had received 5- μ g doses of Enerix had ≥ 10 mIU ml⁻¹ of anti-HBs compared with 98% of those who received 5- μ g doses of Recombivax ($p = 0.06$) or 100% of those who

Table 2 Proportion of people who developed anti-HBs and geometric mean concentrations at 2, 6 and 7 months after initiating hepatitis B vaccination

Vaccine group	Number	% ≥ 1 mIU ml ⁻¹	% ≥ 10 mIU ml ⁻¹	% ≥ 100 mIU ml ⁻¹	Geometric mean
<i>Month 2</i>					
Engerix B 5 μ g	91	51	27	2	1.2
Engerix B 10 μ g	97	61	38	2	2.3
Recombivax 5 μ g	48	81	60	13	9.4
Recombivax 10 μ g	24	83	58	17	14.3
<i>Month 6</i>					
Engerix B 5 μ g	87	69	46	1	3.0
Engerix B 10 μ g	95	79	55	12	7.6
Recombivax 5 μ g	49	94	80	24	30.1
Recombivax 10 μ g	21	100	85	20	59.0
<i>Month 7</i>					
Engerix B 5 μ g	87	92	86	63	176.7
Engerix B 10 μ g	91	95	91	75	325.2
Recombivax 5 μ g	46	98	98	80	570.2
Recombivax 10 μ g	22	100	100	95	744.0

Table 3 Seroconversion and geometric mean titers after immunization of healthy adults with 5, 10 or 20 μ g doses of Engerix B

Reference	Population	Number of vaccinees	Dose (in μ g)	Dosing schedule	Percent ≥ 10 mIU ml ⁻¹	Geometric mean titer (mIU ml ⁻¹)
[12]	Healthy adults	38	5	0, 1, 2	100*	371
[12]	Healthy adults	22	5	0, 1, 6	100*	5865
[15]	Medical students	83	5	0, 1, 2, 6	93	478
[14]	US Navy	56	10	0, 1, 6	84	368
[14]	Healthy adults	69	10	0, 1, 2	93	371
[12]	Healthy adults	74	10	0, 1, 2	99*	386
[12]	Healthy adults	212	10	0, 1, 6	98.6*	1870
[14]	US Navy	48	20	0, 1, 6	84	745
[14]	Healthy adults	70	20	0, 1, 2	98	898
[12]	Healthy adults	96	20	0, 1, 2	100*	300
[12]	Healthy adults	783	20	0, 1, 6	99.4*	974
[14]	Healthy adults <40 years	870	20	0, 1, 6	96	>2000

* ≥ 1 mIU ml⁻¹

received 10- μ g doses of Recombivax ($p=0.14$, chi-square with Yates correction). However, the power to detect differences was low, e.g. <80% for each comparison. The proportion with concentrations of anti-HBs ≥ 100 mIU ml⁻¹ after 3 doses was significantly higher in those receiving Recombivax (80–95%), compared with 63% in those receiving the 5- μ g Engerix regimen. The proportion with ≥ 100 mIU ml⁻¹ in the 10- μ g Engerix group, 73%, was not significantly lower than the Recombivax groups, but power to detect a difference was <60%.

Table 3 demonstrates the geometric mean anti-HBs concentrations over time. The geometric mean continued to increase by about threefold between months 2 and 6 in each group. After the third dose, the geometric mean concentrations were significantly higher in the two Recombivax groups than the 5- μ g Engerix group. Those receiving 10- μ g doses of Engerix had a geometric mean approximately half that of the Recombivax regimens, though this was not statistically significant (unpaired t -test of log-transformed values).

Analysis of volunteer responses according to age, weight and body mass index were performed but were limited by the relatively small proportion of persons 30 years and over (22%), ≥ 170 lb (25%), or with a body mass index (BMI) ≥ 27.7 (17%). No significant differences were detected in the proportion with ≥ 10 mIU ml⁻¹ or GMCs between these groups.

DISCUSSION

This study compares the two recombinant hepatitis B vaccines available in the United States in a prospective, randomized, independent, nonindustry-sponsored protocol. Analysis by an experienced laboratory using standardized techniques for determining quantitative concentration of anti-HBs indicate that all vaccine regimens resulted in protective concentrations of antibody (≥ 10 mIU ml⁻¹) in a high proportion of volunteers. However, Engerix appeared to be somewhat less immunogenic than Recombivax when 5 or 10 μ g of recombinant HBsAg of each vaccine were compared. Recombivax given at half the recommended adult dose (5 μ g) produced anti-HBs in a manner similar to that of 10- μ g doses.

Both recombinant vaccines were well tolerated, with the fewest local symptoms in those receiving only 0.25 ml (5- μ g) doses of Engerix. The group with the highest rate of complaints of pain received Recombivax, 10 μ g in 1 ml. Whether the pain is a reflection of the volume of the injection (1 ml vs 0.5 ml vs 0.25 ml), actual HBsAg antigen per dose, or alum adjuvant per dose (0.125–0.5 mg) is unknown. Degree of pain is an important determinant in whether persons complete a three-dose series.

The main purpose of the study was to determine if a regimen of 5 or 10 μ g of Engerix resulted in a similar

proportion of people achieving anti-HBs of at least 10 mIU ml⁻¹ compared with 5- or 10- μ g regimens of Recombivax. Among those receiving 5- μ g doses of Engerix, 86% achieved this level of antibody, and 92% had ≥ 1 mIU ml⁻¹. Considering those who achieved anti-HBs of at least 100 mIU ml⁻¹, the proportion was significantly less in those immunized with Engerix 5 μ g than in those receiving 5- or 10- μ g doses of Recombivax. Geometric mean concentrations of anti-HBs were significantly less in the 5- μ g Engerix regimen than in either Recombivax regimen. Therefore, the duration of antibody can be expected to be less in this group, though the majority would be expected to respond to either a booster dose of vaccine⁷ or natural infection⁸.

The results of the present study are consistent with previous studies of 5- μ g doses of Engerix in young adults as reviewed elsewhere^{3,9} and with additional studies shown in Table 3. In studies of Engerix 5 μ g IM, 93–100% developed anti-HBs ≥ 1 mIU ml⁻¹ (Refs^{10–12}), while 87% of others developed at least 10 mIU ml⁻¹ anti-HBs. This regimen, while not optimal, may have applicability in some situations. In groups of people where the risk is diffuse and cost is a strong consideration, this regimen may be acceptable. The vaccine was purchased for \$9.00/20- μ g vial, so the cost for three doses of the vaccine is \$6.75, only 7.3% of the cost of the 10 μ g Recombivax regimen.

In the present study, the 10- μ g Engerix regimen, one half the recommended adult dose, resulted in concentrations ≥ 10 mIU ml⁻¹ in 91% of persons and ≥ 100 mIU ml⁻¹ in 75%. This is consistent with other published studies¹³. One study notable for its low anti-HBs response was conducted among healthy adults in the US Navy. Only 84% of those receiving 10- or 20- μ g doses of Engerix had responses ≥ 10 mIU ml⁻¹ anti-HBs. This lower than expected response may have resulted from filling the syringes the night before administration¹⁴.

The results we observed were intermediate to these studies with a 91% response rate ≥ 10 mIU ml⁻¹ after immunization with 10- μ g doses of Engerix. Nevertheless, the cost of this regimen, \$13.50, is only 15% that of 10- μ g doses of Recombivax HB. The Food and Drug Administration has recently approved a dose of 10 μ g of Engerix B for persons ≤ 19 years of age. If this regimen were extended to older groups, it seems prudent that persons at high risk such as health care professionals, sexual partners of patients with hepatitis B virus infection or others with known high risk of hepatitis B have postvaccination anti-HBs antibody performed to ensure adequate response. This testing and revaccination would add considerable cost to this regimen. After a known exposure to hepatitis B, the full recommended dose of either vaccine may be preferable since anti-HBs would be expected to develop more rapidly than with lower doses.

This study confirms the results of earlier studies and the policy in the US military to use the 5- μ g Recombivax regimen in those ≤ 29 years of age^{1–3}. In persons 21–30 years of age immunized with Recombivax HB, 5 μ g IM at 0, 1 and 6 months, 98% of 107 volunteers had detectable antibody with a geometric mean anti-HBs concentration of 1092 mIU ml⁻¹ (Ref.²¹). In another study, approximately 90% of 114 adults immunized with the same regimen developed at least 10 mIU ml⁻¹

anti-HBs with a geometric mean of 349¹. Even lower doses of 2 or 2.5 μ g of Recombivax have also resulted in anti-HBs in 94–100% of young adults^{1,2,15}. In the present study, a regimen of 5- μ g doses resulted in concentrations of anti-HBs almost as high as the 10- μ g Recombivax regimen. The proportion of subjects with protective levels (≥ 10 mIU ml⁻¹) and high concentrations (≥ 100 mIU ml⁻¹) were similar in the two Recombivax groups. The major obstacle for hepatitis B vaccination continues to be the high cost. Even the half-dose regimen costs \$46 per person.

In contrast to a recent retrospective study¹⁶, the present study indicates that Recombivax in full doses of 10 μ g resulted in the expected high proportion of persons with anti-HBs of ≥ 10 mIU ml⁻¹ (100%), and ≥ 100 mIU ml⁻¹ (95%). This is consistent with findings in other young healthy adults^{17,18}. However, studies in adults greater than 40 years of age, who smoke, and have a high body mass index have indicated a much lower rate of seroconversion with plasma-derived vaccines administered IM¹⁹ or intradermally²⁰. Age, smoking, and high body mass index have also been observed to decrease the response to Recombivax or Engerix vaccines^{16,18}.

In this study, Engerix appeared to be less immunogenic than Recombivax on an equal antigen basis. Previous studies have compared 20- μ g doses of Engerix and 10- μ g doses of Recombivax. In one randomized²¹ and one non-randomized study²², 20- μ g doses of Engerix produced higher geometric mean titers than 10- μ g doses of Recombivax, though the differences were not statistically significant. This is consistent with the findings of a retrospective study of health care workers in the United States¹⁶.

Studies comparing 20- μ g doses of Engerix with 20- μ g doses of plasma-derived vaccine have shown a slightly lower GMT and proportion with ≥ 100 mIU ml⁻¹ for Engerix^{12,23,24}. Likewise, intradermal regimens with 2- μ g doses of Engerix have yielded generally poor results^{25,26}. Engerix was less immunogenic than intradermal or intramuscular plasma-derived vaccine²³.

The reason(s) for the lower seroconversion rates and geometric mean titers obtained with Engerix B are unknown. Though both vaccines are produced using recombinant technology, they differ in location of manufacture, physiochemical steps used to purify the product, the amount of residual yeast, treatment with formaldehyde, amount of antigen per milliliter of diluent, and amount of alum for adjuvant. To insure all vaccine was delivered, insulin syringes with no hub (and thus no dead space) were used. A "depot effect" relative to volume of vaccine may relate to differences in response rates.

The study has several limitations. The first relates to sample size. The number of persons vaccinated was limited by the number of susceptible persons. In this population, approximately one-third were found to have anti-HBc before immunization⁵, and additional persons who were entered into the study were later found to have anti-HBc and had to be excluded. The number of persons who could be vaccinated was severely limited by the cost of the vaccine. At \$92 per person, only 25 persons could be randomized into the 10- μ g Recombivax group at a vaccine cost of \$2300, almost half of the entire budget available for vaccines. A group receiving

the standard dose of Engerix B (20 µg) was not included for similar reasons. The relatively small sample sizes may have prevented the detection of possible differences in seroconversion rates between the groups. The sample size also limits the ability to make comparisons with respect to the variables of age, weight and body mass index. The study contains few women, and only 19% were 30 years of age or older. Smoking was not evaluated since few BDF personnel smoke.

In conclusion, use of 5-µg doses of Recombivax in young adults results in a high proportion of vaccinees with protective antibody (≥ 10 mIU ml⁻¹) and should result in significant cost savings for vaccine compared with 10 µg doses of Recombivax. Since the power to detect a lower response in the recipients of the 5-µg doses of Recombivax was limited in this study, additional data should be compiled to determine whether this dose is adequate for young adults in general to possibly allow the vaccine to be labeled by the FDA for use of this dose. Engerix B administered as doses of 10 µg is also immunogenic and costs only one fourth that of the same dose of Recombivax. Engerix administered in doses of 5 µg may not be optimal and cannot be recommended for general use at the present time. Plasma-derived vaccines which are available on the world market for as low as \$1.00/dose may be the most immunogenic and least expensive hepatitis B vaccines^{27,28}.

DISCLAIMER

The views presented here are those of the authors and are not to be construed as official or to necessarily represent those of the Uniformed Services University of the Health Sciences or the Department of Defense.

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- Prevalence Of Sand Fly Fever, West Nile, Crimean-Congo Hemorrhagic
Fever and Leptospirosis Antibodies In Pakistani Military Personnel

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ABSTRACT

To determine the prevalence of antibodies to viral diseases known or suspected to be present in Pakistan, we studied 570 sera from three groups of adults; two of the groups were involved in outbreaks of hepatitis, and the third included men admitted to a hospital for evaluation of febrile illnesses. IgG anti-leptospiral antibody was found in 1-6% of the subjects, with the highest rate in enlisted military personnel hospitalized for febrile illness. One man in the group with febrile illness had significantly elevated IgM anti-leptospiral antibody titers. However, in a group of recruits experiencing suspected non-A, non-B hepatitis, 19 (11%) of 173 had a fourfold rise in IgM antibody to leptospirosis. Antibody to sandfly fever viruses was found in 27-70%. Antibody to West Nile virus was present in 33-41% of subjects. Antibody reactive with Japanese B encephalitis virus was present in 25%, but plaque reduction neutralization tests suggested this to be cross-reaction with West Nile virus. All 212 specimens tested for antibody to Crimean-Congo hemorrhagic fever and Hantaan viruses were negative. This study indicates that diseases known to be prevalent in other areas of Southwest Asia and the Middle East are also prevalent in northern Pakistan and may impact on those traveling or working in this area.

INTRODUCTION

A number of infectious threats which are unique to the Persian Gulf and Southwest Asia have had a major impact on military forces operating in these areas [1]. Sandfly fever virus is transmitted by Phlebotomus papatasi and commonly causes a 2-4 day febrile illness with 1 to 2 weeks of post-febrile asthenia [2, 3]. Sandfly fever had a major impact on Allied Forces in the Persian Gulf in World War II with a rate of 70/1000 troops in 1942 [2]. West Nile virus, a flavivirus transmitted by Culex spp., also causes a febrile illness, often with rash, and occasionally with encephalitis [4]. A related flavivirus, Japanese B encephalitis virus, is present in Southeast Asia and recently has caused severe outbreaks in India [5], but it has not been detected in Pakistan. Crimean-Congo hemorrhagic fever virus, transmitted by Hyalomma spp. ticks in nature and man to man nosocomially, also occurs in Pakistan [6]. Leptospirosis, a febrile illness transmitted through contaminated urine, occurs in Pakistan [7] and India [8]. We performed a serosurvey in 3 groups of patients in Pakistan to determine the seroprevalence of antibodies against these viruses and leptospires and compared our findings with data in the literature about these diseases in Pakistan and neighboring India, Iran, Iraq and Afghanistan.

METHODS

Patients: All patients in the study were men. One group consisted of 212 military recruits from northern Pakistan undergoing training near Mardan, Pakistan, including some who developed jaundice with no serologic evidence of acute hepatitis A or B [9]. Paired sera were collected on these men in October and November 1984. Another group of 192 men, 16-46 years of age, was involved in an epidemic

- of enterically transmitted non-A, non-B hepatitis (hepatitis E) at a service academy in Sargodha, Pakistan, in 1987 [8, 9]. These students and staff were drawn from all over Pakistan. The third group was comprised of 254 patients age 12-75 (mean 28 years) admitted to the Military Hospital in Rawalpindi for evaluation and treatment of acute febrile illnesses between October 1986 and January 1988. Most of these men were from northern Pakistan, and all were enlisted or retired personnel.

Laboratory: Viral serodiagnostic tests were performed by ELISA [10]. Viral antigens were grown in tissue culture cells in roller bottles. Antigen was clarified supernatant maintenance medium collected from infected cultures after cytopathic effects reached 75-100%. The following strains were used: Hantaan virus, prototype strain 76-118; sandfly fever virus Naples, strain 85-055 [11] sandfly fever virus Sicilian (prototype strain-no number); West Nile virus strain EG101; Japanese encephalitis virus, Nakayama strain. Crimean-Congo virus (Strain IbAr10200) was harvested from brains of infected mice as a 10% clarified suspension of brain material in borate saline. Prior to use, virus was inactivated by beta propriolactone [12]. Viral antigen dilution for each assay was determined by checkerboard titrations. A whole cell detergent extract, which is broadly cross-reactive with human antibodies to all known pathogenic serovars of leptospira, was used as leptospiral antigen.

For IgG assays, viral antigens were captured onto the solid phase of PVC microtiter plates by hyperimmune mouse ascitic fluid, which acted as specific immunocapture antibody for each viral agent.

- Dilutions used for each hyperimmune ascitic fluid and its corresponding viral antigen were determined in checkerboard titrations. Leptospiral antigen was coated directly onto PVC plates overnight at 40 C. Sera were tested in duplicate in wells coated as above and against duplicate rows of a mock antigen. Horseradish peroxidase conjugated anti-human IgG (gamma chain specific, prepared in mice, Accurate Chemical and Scientific Co., Westbury, NY) was added, and bound horseradish peroxidase was measured using ABTS (KPL, Gaithersburg, MD), read spectrophotometrically at 410 nm. The adjusted optical density (OD) of the test was obtained by subtracting the average OD of mock antigen-coated wells from specific antigen-coated wells. If this value exceeded a cut-off value obtained by summing the mean and 3 standard deviations of a panel of 5 normal sera, it was considered to be positive. Positive sera were titrated by serial two fold dilutions beginning at 1:100; the last dilution that exceeded the cut-off value was considered the extinction titer.

- IgM antibody was detected using an IgM capture format. Sheep anti-human Mu chain antibody IgM was coated onto PVC plates, and IgM was captured from a 1:100 dilution of sera. Viral- or leptospiral- specific IgM was measured by reacting viral or leptospiral antigen and mock control antigens with captured IgM; those sera containing specific IgM captured the viral or leptospiral antigen, ^{WHICH WAS} and were then measured with an antigen detection system comprised of mouse anti-viral hyperimmune ascitic fluid or rabbit anti-leptospiral antibody, followed by the appropriate horseradish peroxidase-conjugated anti-mouse antibody or anti-rabbit antibody

(KPL, Gaithersburg, MD). Optimal dilutions of viral or leptospiral antigens, virus- or leptospiral-specific antibodies and conjugates were determined by sequential checkerboard titrations. Bound enzyme was quantitated at 410 nm and positive-negative determinations were calculated similarly to the IgG assay: a cut-off value was obtained by summing the mean and 3 standard deviations of the adjusted OD values of a panel of negative sera.

- Titers were determined by serial two⁶fold dilution of sera from
- 1:100, and the last dilution which exceeded the calculated cut⁶off was considered the extinction titer. For both IgG and IgM tests, titers of $\geq 1:200$ were considered positive.

- Plaque reduction neutralization titers for West Nile and Japanese encephalitis viruses were performed for selected sera from patients with febrile illnesses. Human sera were serially four⁶fold diluted in phosphate buffered saline beginning at 1:10 or 1:20, depending upon the volume of serum available for testing. West Nile virus, strain B956, and Japanese encephalitis virus, strain Nakayama, were each diluted to a concentration which would yield approximately 100 plaque-forming units when mixed with equal volumes of diluted human serum. Dilutions of human serum and virus were mixed, held overnight at 4° C, then inoculated onto confluent monolayers of vero cells grown in 12-well plates. Monolayers were overlaid with a nutrient mixture which contained 5% fetal bovine serum, 1% agar, penicillin and streptomycin in 2X Eagle's basic salt solution. Inoculated monolayers were incubated for 4 days at 37°C, stained with neutral red (final concentration 2%) in the same overlay solution. After incubation for an additional 24 hours, plaques were

counted. Results were recorded as the highest serial dilution which neutralized 50% of the plaque dose of approximately 100 plaque-forming units.

RESULTS

Table 1 shows the prevalence of IgG antibody to various pathogens. The prevalence of IgG antibodies to leptospire was low. The highest prevalence, 6%, was seen in the 124 men hospitalized for evaluation of febrile illness. Acute leptospirosis was not clinically suspected in any of the hospitalized men, and in only one subject was a titer of IgM antibody $\geq 1:200$ detected. Likewise, in subjects from the academy, only 3 (1%) subjects had titers of IgG of $\geq 1:200$, and 1 had titers of IgM at 1:800. In contrast, in a group of 212 military recruits that had experienced an outbreak of illness ⁱⁿ which at least 9 men had jaundice, 34 (16%) had IgM titers $\geq 1:200$ on a single specimen. A fourfold rise in antibody titer over one month was observed in 19 (11%) of 173 with paired sera. Of the 9 men with jaundice, only 1 had elevated IgM anti-leptospiral antibodies in the acute serum, and none had IgM anti-HAV or IgM anti-HBc. No convalescent sera were available on these 9 patients.

Antibody to sandfly fever virus of both Naples (SFN) and Sicilian types was prevalent in all three groups. Recruits involved in the outbreak of jaundiced illness and patients with febrile illnesses had the highest prevalence of IgG antibody to sandfly fever Sicilian virus, 70 and 68%, respectively. A much lower prevalence rate was found in those at the service academy in Sargodha, in part because the rate in students was lower than in staff. Antibody to sandfly fever virus, Naples and Sicilian, was present in 12% and 19% of 86

- students 16-18 years of age, respectively, compared with 47% and 60% of 38 staff and faculty ≥ 19 years of age ($p < 0.0001$ both comparisons by Chi square). IgM antibody to SFN was found in 5 (7%) of students and 3 (7%) of staff, while 4 (5%) of students and 5 (11%) of staff at the Academy had IgM to SFS suggesting recent infections with sandfly fever. An increase in the prevalence of antibody to sandfly fever viruses with age was also suggested in patients with febrile illnesses, but only 1.5% of patients had IgM to SFN or SFS, suggesting ^{THAT} these were not an important cause of febrile illness in hospitalized patients.

- Evidence of infection with flaviviruses, in particular West Nile virus, was seen in 35-41% of the three groups. The prevalence increased with age. In those from the service academy, 28% of 86 < 19 years had antibody to West Nile virus compared with 65% of 46 ≥ 19 years of age ($p < 0.0001$). The prevalence of antibody to West Nile virus in patients with febrile illnesses was 41%. Again, a trend toward a higher prevalence in those ≥ 19 years of age was noted but was not statistically significant. A serologic reaction to another flavivirus, Japanese B encephalitis virus, was also seen in 48 (25%) of subjects from the academy and 64 (25%) of patients with febrile illnesses. However, in only 3 (3%) of subjects from the service academy and 9 (11%) of patients with febrile illnesses, were antibody titers higher against Japanese encephalitis virus than West Nile virus. Plaque reduction neutralization titers (PRNT) were done on fifteen selected sera from patients with febrile illness. PRNT titers were higher for West Nile than for Japanese encephalitis virus in all 8 patients whose ELISA titers were

similarly elevated to WN and JE, in all 3 patients whose JE ELISA titer was higher than to WN, and in all 4 patients whose ELISA titer was higher against WN than JE. In 10 (66%) of 15 sera positive for WN by ELISA at $\geq 1:200$, the PRNT titers were $\geq 1:20$. In 7 patients who were negative by ELISA, the neutralization tests were negative for both. The ELISA test appears to be sensitive and specific with no false positive reactions among the 7 specimens which were negative by PRNT. It appears then, that the reactivity by ELISA to JE virus was the result of cross reactions between these antigenically-related viruses.

None of the recruits at Mardan had evidence of antibody to Crimean-Congo hemorrhagic fever virus or Hantaan virus.

Discussion

This study demonstrates that a number of arthropod-borne agents that cause febrile illnesses are prevalent in Pakistan and may be important causes of illness in travelers or others visiting the country. In addition, leptospirosis is present in Pakistan, with reservoirs in rodents, dogs, cattle, buffalo and bandicoots [7]. A seroprevalence as high as 25% (14/56) has been reported in patients hospitalized in Karachi, Pakistan [7]. In neighboring Afghanistan, 17 (1.4%) of 1,214 sera were positive by the macro slide agglutination test for leptospirosis [13]. This low prevalence is consistent with findings in two of our study populations. However, in military recruits, the finding of significant titers of IgM in 16%, including 11% in whom fourfold rises in titer were detected, suggests that some illnesses experienced in these military recruits were due to

acute leptospirosis, though hepatitis E is suspected to be the cause of illness in many of these men.

Because of the potential for debility in large numbers of non-

- immune hosts, sandfly fever has long been a disease of military importance [2, 3, 14]; Eitrem, 1990 #374. In the Khyber Pass area of Pakistan-Afghanistan and in the plains southeast of Teheran, Iran,
- sandfly fever viruses have been isolated from patients with febrile
- illnesses and from pools of sandflies [15]. Serologic studies of patients in Karachi, Pakistan revealed 2.7% of 75 sera had a positive
- neutralization test for sandfly fever Sicilian, while 9.3% were
- positive for sandfly fever Naples [16] Antibodies to sandfly fever virus were detected in only 7 (0.6%) of 1,214 sera by
- hemagglutination inhibition test from 4 widely separated villages in
- Afghanistan [13]. In Iran, the prevalence of antibody to sandfly fever
- Naples and Sicilian, by plaque reduction neutralization test, was 17
- and 25%, respectively. Antibody to another strain, Karimabad, was much higher (66%) [17]. Antibody to Karimabad was not found in sera
- from Pakistan [16, 18]. The prevalence of antibody to sandfly fever has been noted to increase rapidly with age [19]. The lower
- prevalence ^{THAT} we observed in students compared with staff and faculty
- at the academy may relate to ^{THE SHORTER} less duration and ^{LOWER} intensity of exposure
- in military activities, ^{AMONG THE STUDENTS} less exposure ^{IN THEIR} due to ^{AREAS OF} geographic origin, since
- students come from all over Pakistan, or less exposure relating to
- socio-economic factors.
- A recent study using an ELISA test for sandfly fever Naples, Sicilian, and Toscano found that an ELISA test system very similar
- to the one we used was very sensitive but not strain specific with

- regard to plaque reduction neutralization tests in blood bank sera from Greece. A number of specimens which were positive by ELISA and indirect immunofluorescence test were negative by PRNT, suggesting a possible cross reaction with other strains of sandfly fever virus which have not yet been identified from man [20].

West Nile virus usually causes a mild disease in children but may cause severe disease, including encephalitis, in adults. West Nile virus was isolated from plasma of 2 of 173 patients with febrile illnesses in Rawalpindi [21-23]. Five additional isolates were obtained from 144 patients with fever in Lahore [24]. The clinical course of these patients was characterized by fever, severe headache, retrobulbar pain, myalgias, lymphadenopathy and leukopenia. Rash was present in some patients [25].

- Seroprevalence studies have revealed neutralizing antibody to West Nile virus in 33% of residents in a semi-arid area in Punjab Province and in 38% in the Changa Manga National Forest, also in Punjab Province [24], while in Karachi, the seroprevalence was 50% [26]. All three surveys found an increasing prevalence with age. A lower prevalence, 11.6%, was found in another study [16], while in the mountains of northern Pakistan, no antibody was detected in a survey of 93 people [25]. West Nile virus has been isolated from Culex mosquitoes near Rawalpindi [22] and Lahore [27]. Serologic evidence of West Nile virus was found in about one-third of all birds and buffaloes studied near Lahore [24]. In Afghanistan, the prevalence of antibody to West Nile virus by hemagglutination inhibition studies in four villages ranged from 1-97% [13]. The seroprevalence of West Nile virus neutralizing antibodies in 13

communities in Iran ranged from 0-96%, depending on the region [17].

Even though 25% of the subjects in the present study had antibody reactive with Japanese B encephalitis (JE) virus, it is unlikely that JE is prevalent in Pakistan. Nearly all sera had higher titers against

- West Nile virus. Others have noted ^{THAT} neutralization tests indicate
- antibody to West Nile [13, 26, 27] In subjects with equal ELISA
- titers against both West Nile and JE, we, and others [13], have found
- neutralization antibody titers to be consistently higher against West Nile virus. Furthermore, no viral isolates of JE have been made in
- Pakistan [25] The disease is prevalent in parts of India, but has not been reported in areas of India near Pakistan [5, 28, 29]. Even though the main vector for JE, *Culex tritaeniorhynchus*, is a very common mosquito throughout the region [25, 27], domestic swine, which act as amplifying hosts, are proscribed by Islam. Cattle and buffalo are not thought to be good amplifying hosts for JE [29, 30].

- Other flaviviruses that might give a serologic cross ^{reaction} with West Nile virus are not prevalent in Pakistan. Yellow fever is not present in Pakistan, nor was yellow fever vaccine given to those studied. Dengue virus caused epidemics in Calcutta in 1966 and in Delhi, India in 1982 and 1988 [31-33] but has only once been isolated from a patient in Pakistan [25]. This was in Lahore, which is near the Indian border. The vectors for dengue, *Aedes aegypti* and
- *Aedes albopictus*, are present focally in Pakistan [25] A low prevalence of antibody reactive with dengue has been found in some
- surveys, but this may represent a cross ^{reaction} with other flaviviruses [16, 26]. Additional flaviviruses, such as Russian

spring-summer encephalitis virus and Kyasanur Forest disease, occur in neighboring countries, but have not been isolated in Pakistan [25, 34].

Crimean-Congo hemorrhagic fever (CCHF) occurs in Pakistan and has caused at least 50 cases that have reached medical attention since 1976. Infection has occurred in those working with animals, and in two instances, a total of 13 medical staff were infected when surgical operations were performed on patients thought to have bleeding peptic ulcer disease. In each outbreak, a surgeon died of nosocomial infection [6, 25], (unpublished report by Burney, 1987).

- The vector is a tick of the genus Hyaloma. Two isolations of CCHF virus were made from ticks collected in the Changa Manga Forest in 1965 [6, 25]. We found no serologic evidence of CCHF in our present surveys. Other serosurveys in Pakistan, a border area in Kashmir State in India, and Iran have also found a very low prevalence of antibody, suggesting a low infection rate or low survival rate in those infected [16, 25, 35]. No isolations had been made in India as of 1986 [16, 25, 35], but outbreaks have occurred in other countries bordering Pakistan, such as Iraq and the Soviet Union [36, 37].

- This study indicates that leptospirosis, WN virus and sandfly fever viruses frequently cause infections in northern Pakistan, which includes the capital city of Islamabad. While a low incidence
- of sandfly fever, Crimean-Congo hemorrhagic fever, and West Nile virus infections were noted in US Marines in Operation Desert Storm/Desert Shield, this probably resulted from a ^{LESSER} low exposure, due
- to the winter time frame and from good personal protective ^{on} measures [38]. With many foreign visitors to the Persian Gulf and

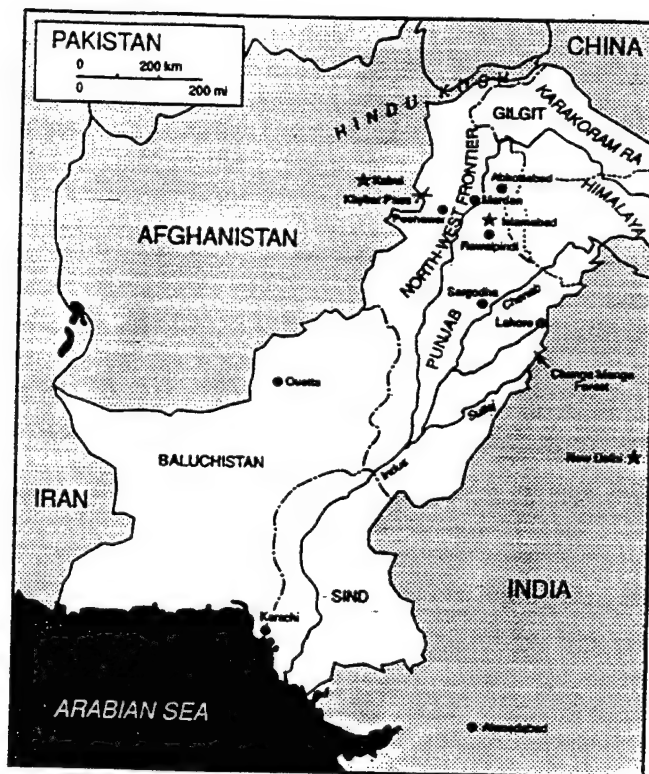
- southwest Asia who may be susceptible, the occurrence of febrile illness should bring these disease possibilities to mind [39].

Table 1. Prevalence of IgG antibody at titers of $\geq 1:200$ to Leptospirosis and selected viruses in 3 military populations in Pakistan

	Recruits	Academy	Patients with Fever
Antigen	N = 212	N = 192	N = 254
Leptospirosis	3 (1%)	3 (2%)	7 (6%)*
- Sandfly Fever Naples	75 (35%)	53 (27%)	136 (54%)
- Sandfly Fever Sicilian	149 (70%)	64 (33%)	172 (68%)
West Nile	75 (35%)	63 (33%)	105 (41%)
Japanese Encephalitis	Not Tested	48 (25%)	64 (25%)
Crimean-Congo	0 (0%)	Not tested	Not tested
Hemorrhagic Fever			
Haantan Virus	0 (0%)	Not Tested	Not tested

*Only 124 sera were tested

Figure one. Map of Pakistan depicting sites of major interest from the study and surrounding countries.



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Epidemic Hepatitis E in Pakistan: Patterns of Serologic Response and Evidence that Antibody to Hepatitis E Virus Protects against Disease

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IgM and IgG anti-hepatitis E virus (HEV) patterns were determined in sera collected during a hepatitis outbreak in Pakistan. HEV infection was detected serologically in 122 patients. IgM anti-HEV was detected in specimens collected up to 2 weeks before and 5-7 weeks after hospitalization in 91% and 100%, respectively, of 122 HEV-infected patients. IgG followed a similar pattern. Peak antibody titers appeared 2-4 weeks after hospitalization. At 20 months after hospitalization, IgM anti-HEV was not detected in any of 33 patients; IgG was found in all. IgG anti-HEV appeared to be protective in contacts of patients. This study confirms HEV as the cause of the outbreak, quantifies IgM and IgG anti-HEV responses, provides evidence that IgG anti-HEV protects against hepatitis E, and demonstrates that IgG anti-HEV persists, but at diminished titer, after infection. Hepatitis E in young adults is the result of primary infection with HEV and, if reinfection occurs, it does not commonly cause serious illness.

Hepatitis E virus (HEV) is enterically transmitted and causes sporadic and epidemic cases of hepatitis most often in Africa and Asia but also in Mexico [1]. Outbreaks of non-A, non-B hepatitis (presumably hepatitis E) have occurred in Pakistan [2, 3], and travelers returning from Pakistan, India, Nepal, and Mexico have developed hepatitis E [4, 5]. In the spring of 1987, an outbreak of non-A, non-B hepatitis, in which 133 persons were hospitalized, occurred at a college (high school) in Sargodha, Pakistan [6]. Detection of HEV by immune electron microscopy (IEM) in fecal specimens from 10 persons and a serologic response detected by IEM in another person with symptoms of hepatitis were strong evidence that at least some of the cases were caused by HEV [7].

Cynomolgus monkeys developed hepatitis after inoculation with HEV-containing feces (strain SAR-55) from a patient hospitalized with hepatitis during the outbreak. HEV was recovered from the bile and feces of the monkeys. Using

virus excreted from one of these monkeys, virtually all of the viral genome was cloned [8]. The second open reading frame (ORF-2) of this HEV was expressed in baculovirus and used to develop an ELISA for hepatitis E [9]. We used this ELISA for further investigation of the Sargodha hepatitis outbreak, including determination of the infection rate in contacts of cases, the pattern of IgG and IgM anti-HEV over 20 months, the relationship of these antibodies to disease, and the correlation between the patterns of HEV in feces and anti-HEV in serum.

Methods

Collection of specimens. The epidemiologic aspects of the outbreak have been reported [6], as has the detection of HEV in feces [7]. On 20-22 March 1987, investigators visited the school and hospital in Sargodha, Pakistan, where hepatitis was occurring and collected sera and feces from hospitalized patients. In addition, an assembly was held at the school to explain the situation to students and staff and to obtain sera and feces from these nonhospitalized contacts, especially those who reported symptoms compatible with hepatitis, such as dark urine, light-colored feces, and scleral icterus. At a repeat visit to the hospital on 18 April 1987, serum and fecal samples were collected from new patients with hepatitis and once again from previously admitted patients. Samples were also collected from contacts of these patients. Sera was collected again on 10 December 1988.

For the 192 persons studied, 253 serum samples were available: 132 from March 1987 (73 hospitalized and 59 nonhospitalized patients), 76 from April 1987 (all patients hospitalized at time of blood collection or soon thereafter), and 45 from December 1988 (33 patients had been hospitalized and 12 had not). Of these 253 samples, 18 were pairs from March and April 1987 and 45 were pairs from March or April 1987 and December 1988. Serum samples were available from all 3 collection days for only 4 persons.

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Informed consent was obtained from patients and contacts. Guidelines for the ethical use of human subjects at Uniformed Services University of the Health Sciences and Army Medical College were followed.

Opinions and assertions contained herein are those of the authors and do not necessarily represent those of the Department of Defense or the Uniformed Services University of the Health Sciences.

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Table 1. Diagnostic criteria for viral hepatitis in hospitalized patients during an epidemic of hepatitis.

Hepatitis type	Cases, no.	%
Acute hepatitis E		92.4
IgM anti-HEV	120*	
IgG anti-HEV seroconversion	2	
HEV in feces	1	
Acute hepatitis B	1*	0.8
Undiagnosed	9	6.8
Total	133	100.0

* 1 of 2 patients with IgM anti-HBc, indicating acute hepatitis B, also had IgM anti-HEV and is counted as a case of hepatitis E.

ELISA for anti-HEV. Anti-HEV ELISA was done as described previously [9]. Each well of a flexible microtiter assay plate (Falcon; Becton-Dickinson Labware, Oxnard, CA) was covered with 100 μ L of carbonate buffer (pH 9.6) containing 5×10^4 lysed SF9 insect cells expressing ORF-2 of the HEV genome. After incubation for 2 h at 37°C, the plates were blocked for 1 h at 37°C with PBS (pH 7.2) containing 0.5% gelatin and 10% fetal bovine serum. Serum samples were diluted in blocking solution and assayed at dilutions of 1:100, 1:1000, and 1:10,000. Peroxidase-conjugated goat anti-human IgG or IgM (Organon Teknica Cappel, West Chester, PA) was used as the detector antibody.

The following criteria, similar to those previously used in ELISAs [9–11], were used to determine the titer of anti-HEV in each serum sample. Samples were considered positive for anti-HEV when the optical density was ≥ 0.35 at a test dilution of 1:100, ≥ 0.2 at 1:1000, and ≥ 0.15 at higher dilutions. These values were ≥ 4 SD above the mean value determined for 19 people not admitted to the hospital. An anti-HEV titer was the greatest dilution for which these requirements were fulfilled. All tests were done without knowledge of the clinical status of the patients.

Statistics. In calculating geometric mean reciprocal titers (GMTs) of anti-HEV, patients with titers of <100 were included and arbitrarily assigned a value of 1. Proportions were compared using χ^2 with Yates's correction or partitioned χ^2 .

Results

Between 1 March and 22 April 1987, 133 men with a clinical diagnosis of hepatitis were admitted to the hospital (table 1). None of these patients had IgM antibody to hepatitis A. Hepatitis B surface antigen was detected in 6 patients, and anti-hepatitis B core antigen (HBc) was detected in 26, but only 2 of the 133 patients had IgM anti-HBc, indicating acute hepatitis B [6]. All patients were tested for anti-HEV. IgM anti-HEV was detected in sera obtained from 119 patients during March and from 1 patient in April. One of these patients also had IgM anti-HBc, indicating infection with both HEV and HBV. A ≥ 10 -fold rise in IgG anti-HEV (seroconversion) was documented between March 1987 and December 1988 in 2 additional patients, indicating infection, possibly at the time of the epidemic. Anti-HEV was not detected in serum samples of 1 patient who later had HEV

detected in a feces sample by IEM, a finding later confirmed by animal inoculation (data not shown) [7]. Thus, of the 133 patients hospitalized for hepatitis, a diagnosis of hepatitis E was made in 123 (92%), including 1 patient with both hepatitis B and E infections, and 1 patient was diagnosed with acute hepatitis B.

Biochemical evidence of hepatitis (elevated alanine aminotransferase [ALT]) was documented in 130 (98%) of the patients, while 111 (83%) had ALT values ≥ 1.5 times the upper limit of normal. The 3 patients with normal ALT values at the time of the initial serum collection included 1 patient with IgM anti-HEV, 1 with HEV in feces, and 1 of 9 patients who did not have a confirmed diagnosis of viral hepatitis. Therefore, clinical diagnosis correlated well with biochemical evidence of hepatitis.

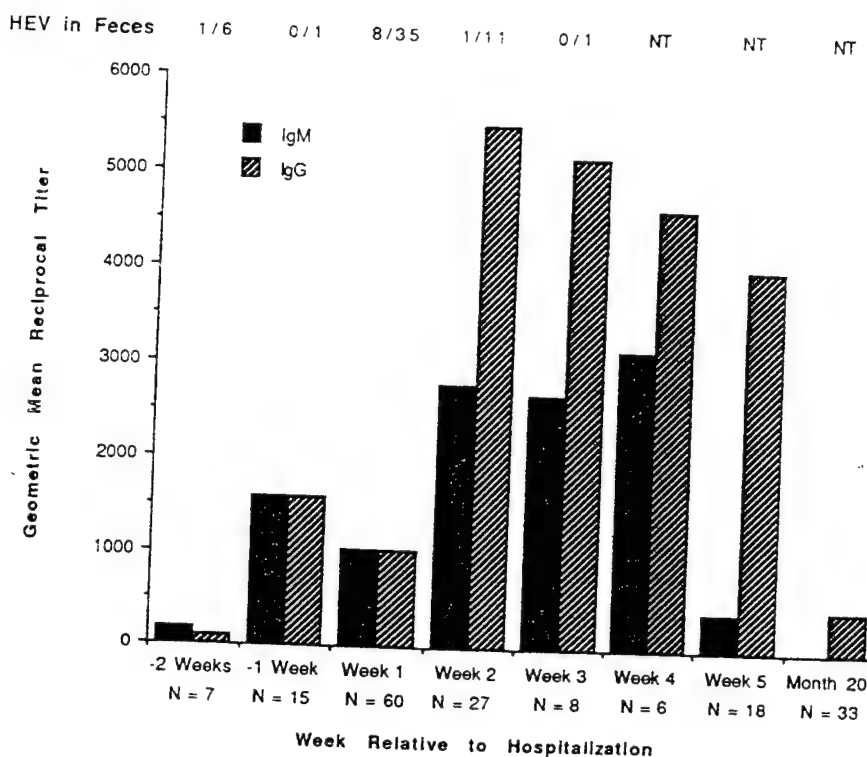
For the 9 hospitalized patients not diagnosed with viral hepatitis, only a single serum sample collected in March was available. In 6 of these patients, sera were collected 9–29 days before admission. Two patients had IgG anti-HEV, including 1 with a titer of 1:10,000. ALT values were normal in 4 patients and mildly elevated in 4 others. Only the patient with a high IgG anti-HEV had an ALT value ≥ 1.5 times normal. Fecal samples from 7 of the 9 patients were negative for HEV. Therefore, the inability to diagnose acute viral hepatitis may indicate that these patients had either early or mild disease or a nonviral hepatitis illness.

Because sera collected in March and April were obtained from patients at various times before or during hospitalization, it was possible to study the serologic patterns relative to the week of admission (table 2). IgM anti-HEV was detected in 2 of 8 serum samples obtained 3–5 weeks before admission, in 5 of 7 obtained during week 2 before admission, and in all 15 collected the week before hospitalization. The titers were $\geq 1:1000$ in all but 1 sample. IgM anti-HEV was detected in 96 of 101 samples obtained within the first 4 weeks after hospital admission (the 5 hospitalized patients in whom IgM anti-HEV was not detected during the first 4 weeks of admission are described above). IgM anti-HEV continued to be detectable in all 18 serum samples obtained 5–7 weeks after admission, but the titers in 10 were lower than in corresponding sera collected 1 month earlier. At 86 to 92 weeks

Table 2. Comparison of IgM and IgG anti-hepatitis E virus in serum samples relative to week of admission to hospital.

Weeks	IgM anti-HEV		IgG anti-HEV	
	No. positive/ no. tested	%	No. positive/ no. tested	%
–5 to –3	2/8	25	1/8	13
–2	5/7	71	5/7	71
–1	15/15	100	15/15	100
+1 to 4	96/101	95	98/101	97
+5 to 7	18/18	100	18/18	100
+86 to 92	0/33	—	33/33	100

Figure 1. Semiquantitative analysis of IgM and IgG antibody to HEV in patients hospitalized with hepatitis E. Number of feces positive for HEV/number examined by immune electron microscopy [7] for each time point listed at top. NT = not tested; N = number of patients.



(~20 months) after admission. IgM anti-HEV was no longer detectable in any of the 33 serum samples available from previously hospitalized patients.

IgG anti-HEV was detected in 119 serum samples from patients who were in the hospital or were later hospitalized. Only 1 of 8 samples obtained 3–5 weeks before admission had a titer $\geq 1:100$. However, IgG anti-HEV was detected in 5 of 7 samples obtained 2 weeks before admission, in all 15 collected during the week before admission, and in 98 of 101 obtained within the first 4 weeks of hospitalization. In addition, all 18 serum samples obtained 5–7 weeks after admission had detectable IgG anti-HEV, including 11 that had detectable antibody at 10-fold higher dilutions than in corresponding sera obtained 1 month earlier. Approximately 20 months after admission, IgG anti-HEV was still detectable in all 33 serum samples obtained from previously hospitalized patients.

Figure 1 shows a semiquantitative analysis of IgG and IgM anti-HEV in sera obtained 2 weeks before to 20 months after hospitalization. IgM anti-HEV was detected in patients 2 weeks before admission at a GMT of ~ 193 . IgM anti-HEV appeared to peak during weeks 2–4 of hospitalization, with GMTs of ~ 3000 . By weeks 5 and 6 after admission, the GMT of IgM anti-HEV had fallen below 500 and could not be detected in any specimen by month 20. IgG anti-HEV was detected at a GMT of ~ 100 2 weeks before admission, peaked at a titer of >5000 during week 2 after admission, and remained detectable at a GMT of almost 500 20 months later.

The inclusion of contacts of patients with hepatitis pro-

vided special opportunities for study. Because some of these contacts were selected on the basis of symptoms suggesting hepatitis, selection bias was built into the group. Not surprisingly, of the 75 contacts of hospitalized patients who were prospectively studied in March and followed subsequently, 16 (21%) were later hospitalized with hepatitis. These are included among the cases described above and in table 1.

However, 59 (79%) of the contacts were not hospitalized (table 3). IgM anti-HEV was detected in 13 (23%) of these contacts, IgG without IgM anti-HEV was detected in 30 (50%), and there was no detectable anti-HEV in 16 (27%). A second serum sample obtained 20 months later was available from 12 of the 59 nonhospitalized contacts, including 10 who had no detectable IgM anti-HEV at the time of the outbreak. Two of these 10 persons had developed IgG anti-HEV, and 1 had an increase of IgG anti-HEV from 1:100 to 1:1000 20 months later, suggesting exposure to HEV during or after the time of the outbreak. No additional cases were detected by IEM of fecal samples from 36 of these 59 nonhospitalized contacts. Therefore, of the 75 contacts of hospitalized patients, 10 hospitalized and 16 nonhospitalized contacts had serologic evidence of infection (IgM or IgG anti-HEV seroconversion), giving a total infection rate of at least 35% among the contacts. Assuming that those who developed IgG anti-HEV were infected at the time of the epidemic, the total number of documented HEV infections was 139 (including the patient with acute hepatitis B and HEV) (table 3).

Some persons exposed in the outbreak appear to have been protected from illness requiring hospitalization because

Table 3. Results of diagnostic tests for hepatitis E in 192 patients and contacts.

Subject, no.	Serology			No evidence of HEV
	IgM anti-HEV	IgG anti-HEV seroconversion only	HEV in feces	
Patient hospitalized with jaundice, 117	112*	0	1	4†
Contact				
Hospitalized later, 16	8	2		6
Not hospitalized, 59	13	3		43
Total, 192	133 (69.0%)	5 (2.5%)	1 (0.5%)‡	53 (27.0%)

NOTE. Data are no. unless indicated otherwise.

* Includes 1 patient with acute hepatitis B who also had IgM anti-HEV.

† Includes 1 patient with acute hepatitis B without evidence of HEV infection.

‡ Confirmed by animal inoculation with patient's HEV-infected feces.

of preexisting anti-HEV. Of the 75 contacts of patients who were followed prospectively, none of 30 with only IgG anti-HEV in initial serum samples were admitted to the hospital with hepatitis, compared with 8 (33%) of 24 in whom no anti-HEV was detected ($P = < .001$ by χ^2). Eight (38%) of 21 persons in whom IgM anti-HEV was detected in initial serum were also hospitalized.

Serologic testing confirmed HEV infection (IgM anti-HEV titers $\geq 1:1000$) in 9 of 10 patients in whom HEV was detected in feces by IEM. In 1 person, a low concentration of HEV-like particles was detected in stool, but IgM and IgG anti-HEV were not detected in the single serum sample obtained 2 days after admission. However, a diagnosis of hepatitis E in this patient was confirmed by transmission of hepatitis E to cynomolgus monkeys and chimpanzees following inoculation with the patient's feces (data not shown). Eight of the 10 fecal samples in which HEV was detected by IEM were obtained within the first 2 days after admission (figure 1). Among the 86 persons in whom HEV was not detected in feces by IEM, HEV infection was confirmed by IgM anti-HEV in 50, including 40 (80%) of 50 hospitalized and 10 (28%) of 36 nonhospitalized patients.

Discussion

This new ELISA for HEV [9], using an antigen produced in insect cells by recombinant technology from HEV recovered in the Pakistan outbreak, appears to be quite sensitive for detecting IgM and IgG anti-HEV in sera of patients and contacts. This assay has confirmed acute or recent HEV infection in 123 (92%) of 133 patients admitted to the hospital and at least 139 infections among 192 persons studied. This contrasts with only 10 documented infections of HEV demonstrated by IEM studies of feces from 96 of these persons [7]. We also determined the pattern and kinetics of IgM and IgG anti-HEV responses, including the documentation of IgG antibody almost 2 years after infection. Furthermore, we have demonstrated that subclinical disease occurred in at

least 16 (27%) of 59 nonhospitalized contacts of persons infected with HEV. We speculate that still others were protected because of the presence of preexisting antibody.

This is one of the first sensitive and practical assays for IgM anti-HEV. Others [12, 13] have reported the detection of anti-HEV by ELISA with antigens produced in *Escherichia coli*. In those studies, IgM anti-HEV was detected in 40%–86% of 22 non-A, non-B patients. In a study of patients with hepatitis in Hong Kong [14], the rate of detection of IgM anti-HEV by ELISA with *E. coli*-derived antigen was 5.8%, which was not much higher than the 3.9% rate of IgM anti-HEV found in healthy subjects in the study. Using a portion of *E. coli*-derived HEV ORF-2 in a Western blot, IgM anti-HEV was detected in 23 (92%) of 25 pediatric cases of non-A, non-B hepatitis and in 3 (8%) of 39 controls in Sudan [15] and in 58 (53%) of 110 Egyptian children with non-A, non-B acute hepatitis [16]. Similar results have been reported in epidemics of hepatitis E in Somalia [17].

In the present study, IgM anti-HEV was detected in 96% of 125 sera obtained up to 9 days before admission to the hospital. The assay also was specific in that IgM anti-HEV was not detected in any serum obtained 20 months after the epidemic from 33 previously hospitalized patients or in 12 nonhospitalized but infected contacts. In 1 patient in whom anti-HEV was not detected by IEM in the first serum sample but was detected 1 month later [7], the results of the ELISA were consistent with IEM results: Anti-HEV was not detected in the first serum, but IgM and IgG anti-HEV titers of 1:1000 were detected in the later specimen. The effectiveness of this assay in detecting IgM in patients in other Asian or Mexican locations remains to be determined, but it was able to detect anti-HEV with a high degree of sensitivity in cynomolgus monkeys infected with the most genetically diverse isolate of HEV identified to date, the Mexican strain [9], and there is only 1 known serotype of HEV [7, 18, 19].

Using this assay, we determined the kinetics of anti-HEV responses in a relatively large number of patients. IgM anti-HEV was consistently detected up to 2 weeks before hospital-

ization and for at least 6 weeks after admission. IgG anti-HEV was also present 2 weeks before admission and peaked ~2 weeks later but remained detectable in low to moderate titers for at least 20 months in all patients with detectable antibodies during acute illness. IgG anti-HEV has been detected in experimentally infected animals for at least 6 months and in a volunteer for at least 2 years after infection [18, 20].

This assay for anti-HEV in serum adds a new perspective to the relationship between anti-HEV in serum and HEV in feces. In this study, we detected both IgG and IgM antibodies in 9 of 10 serum samples collected from patients on the same day that HEV was detected in their fecal specimens by IEM. This early detection of anti-HEV further attests to the sensitivity of the assay. The relationship between appearance of antibody in serum and the clearance of virus from serum, bile, and feces needs further study.

A seroepidemiologic survey in Hong Kong found an increasing prevalence of IgG anti-HEV with age, from 3% in patients <10 to 30% in those >30 years old [14]. There was a correlation with antibody to hepatitis A virus, the prevalence of which also increased with age, suggesting parallel exposure to both hepatitis viruses. A similar increase in prevalence of anti-HEV with age was also suggested by data from Somalia [17]. Our finding of a higher prevalence of IgG without IgM in nonhospitalized persons >18 years old than in those ≤18 is consistent with the data from Hong Kong and Somalia. The results of these studies suggest the accumulation of HEV infections over time or, alternatively, diminishing importance of HEV in recent years. Additional studies are required to resolve this question.

Finally, our data suggest that the presence of preexisting IgG anti-HEV prevents hepatitis E. Among contacts of patients with hepatitis, 33% of those with no IgG or IgM anti-HEV when initially studied were later hospitalized with hepatitis E. In contrast, no patients with IgG but without IgM anti-HEV were later hospitalized. This has two important implications. First, hepatitis E in young adults is apparently the result of a primary infection with HEV, not a reinfection. This conclusion is supported by the detection of IgM anti-HEV in virtually every case. Second, the data suggest that hepatitis E may be prevented by immune serum globulin containing anti-HEV or by an HEV vaccine. This bodes well for future immunoprophylaxis of hepatitis E.

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Seroprevalence of Hepatitis B Virus

Among School-Aged Children in the Stann Creek District
of Belize, Central America

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ABSTRACT

Adults in the Stann Creek District of Belize have a high prevalence of hepatitis B virus (HBV) infection but the age of onset of these infections is unclear. We conducted a seroprevalence study of hepatitis B markers among Stann Creek school-aged children of different backgrounds to provide information for planning a hepatitis B vaccine program. After informed parental consent, 587 students from five schools were tested for antibody to hepatitis B core antigen (anti-HBc) and hepatitis B surface antigen (HBsAg). The overall prevalence of hepatitis markers was high: 43.3% had anti-HBc and 7.7% had HBsAg. There was marked variation of anti-HBc by school and by the predominant ethnic groups attending those schools. Maya had the highest prevalence (76%), followed by Mestizo (50%), Garifuna (37%), and Creole (25%). Children less than 9 years of age attending the rural primary schools (mostly Mayan and Mestizo) ~~had significantly higher prevalence of anti-HBc than did children attending the urban primary~~ school (mostly Garifuna and Creole) ($p < 0.05$). Anti-HBc was found in 42% and 36% of students at the two high schools. Of the five schools tested, only at the urban primary school did anti-HBc positivity increase with increasing age. Based on an analysis of the cost of serologic screening before immunization compared with mass vaccination, pre-immunization serologic screening resulted in vaccine program cost savings in four of the five schools. Because most children in the rural areas contract hepatitis B before entering school, immunization against HBV should be integrated into the routine infant immunization program.

Hepatitis B virus (HBV) endemicity varies markedly in Latin American countries depending on local occupational, socioeconomic, racial, and other factors.¹ In the Central American nation of Belize, studies of adults confirm a wide variation in HBV prevalence by ethnicity (from 5% anti-HBc reactivity among Mestizo to 56% among Garifuna) and by district, with the highest prevalence of infection in the southern districts of Stann Creek (67%) and Toledo (33%).^{2,3} Spurred by this high HBV seroprevalence in Stann Creek adults, the feasibility of a hepatitis B immunization program focused on the children of this district was considered.

Several immunization strategies for HBV control have been proposed. In settings where infections occur at early ages, immunization of newborns and infants may be the most effective means of controlling HBV.⁴ In settings where HBV exposure occurs in later childhood, school-based vaccination programs may prevent HBV transmission.⁵ Finally, selective immunization programs targeting high risk groups can also be effective in reducing the risk of infection if diligently applied.⁶ The strategy which is most appropriate depends on the age of HBV infection and other epidemiological parameters.

In populations with a high prevalence of HBV, prevaccination screening may lower vaccine program costs by avoiding unnecessary vaccination of immune or carrier individuals. Knowledge of HBV frequency and distribution is necessary to evaluate the costs and potential benefits of prevaccination screening. Despite the clear risks of hepatitis B infection in Stann Creek district, the epidemiology of HBV among children of that district was unknown. To provide this information, we conducted a study in the Stann Creek district of Belize, evaluating HBV prevalence among school-aged children of different ages, ethnic groups and geographic

locations. We also sought to determine the local cost-effectiveness of prevaccination HBV screening. At the same time, we evaluated the frequency of anemia and malaria in these students.

MATERIALS AND METHODS

The study was approved by the Human Use Review Committees of the Belizean Ministry of Health and of the Uniformed Services University of the Health Sciences and was conducted in the spring of 1995. After preliminary meetings with school board officials, principals and parents to inform them of the study, children/young adults attending three primary and two high schools in the Stann Creek district were invited to participate. Questionnaire data (which included identifying demographic and medical history information) and informed parental consent were obtained for each volunteer. An 8-ml venous blood sample was then collected and labeled with a unique identification number.

Serum samples were tested for antibody to HBV core antigen (anti-HBc) by Corzyme[®] (Abbott Laboratories, Abbott Park, IL). Samples repeatedly positive for anti-HBc were tested for hepatitis B surface antigen (HBsAg) by Auszyme[®] (Abbott). All tests with positive results were repeated for confirmation. In addition, thick-smears of blood were microscopically examined for malaria parasites and a screening test for anemia was performed using a spun hematocrit method. All hematocrit values < 37% were repeated for confirmation. Data analysis was performed using Epi Info 6.0.⁷

This study was designed to evaluate the cost effectiveness of prevaccination HBV screening by quantification of three variables: the cost of vaccination, the cost of screening for

susceptibility, and the expected prevalence of immune individuals in the group.^{3,4,8} The cost of vaccination includes the vaccine cost (\$13.50 for three pediatric doses of Engerix B, Smith Kline Beecham) plus an estimated vaccine administration cost (\$10.00/person to administer 3 doses). The cost of screening (\$7.00/person) is based on the actual study costs and includes cost of anti-HBc test kits (\$3.75/test), venipuncture supplies (\$0.70/person), and administrative costs (\$2.55/person tested which includes labor for venipuncture and lab testing, transportation, per diem and overnight lodging). Screening cost does not include the cost of confirmatory testing because of the very high concordance in confirmatory testing. Average cost per person is based on the total cost divided by the total number of children surveyed. All costs are in U.S. dollars.

Study population

Two rural primary schools (rural primary A and rural primary B), one urban primary school, and two urban high schools (high school 1 and high school 2) were included in the survey. Of the three primary schools sampled, one is in an urban area in the northern part of the district, one in a southern Mayan community, and one in a southern community largely comprised of immigrant farm workers from other Central American countries. Although both high schools are located in semi-urban areas (one in the northern and one in the southern part of the district), some students commute to school from rural towns. High school 1, the larger of the two high schools, provides a college preparatory program and is the source of many of the 19-22 year old students in our survey. Because of its large size and our desire to sample older as well as younger subjects, only students from the upper two grades and the college preparatory program at high school 1 were invited to participate in the study.

Stann Creek district has an ethnically/racially diverse population (as does Belize as a whole). The total population of Stann Creek in 1991 was 18,085 with 5-24 year old individuals comprising approximately 45% of the population (1991 population census data). There are four main ethnic groups in the Stann Creek district: Garifuna (descendants of African slaves and Carib Indians), Creole (of mixed African ancestry), Mestizo (of Spanish and Mayan origin), and the Mopan and Ketchi Maya (the indigenous people of Belize).

RESULTS

Hepatitis B prevalence

There were 587 students tested from five schools, age range 4-22 years (median age of 13 years). The ethnic composition of the study participants in comparison to the total population of Stann Creek is shown in Table 1. The Maya were over-represented in our survey because one of the rural primary schools studied was in a Mayan community. Eighty-one percent of children attending the predominantly Mayan primary school A and 48% of children attending the urban primary school were tested; 47% of students in the three upper grades invited to participate at high school 1 and 64% of students at high school 2 were tested. In contrast, rural primary school B had a 12% response rate in large part due to difficulties in contacting parents to complete consent forms.

Table 2 shows the results of anti-HBc and HBsAg testing by sex, age, ethnicity and school. Two hundred and fifty four students (43.3%) were reactive for anti-HBc, with marked variation between schools and between the predominant ethnic groups attending those schools.

Forty five students (7.7%) had HBsAg. Rate of anti-HBc positivity was significantly higher in males (52%) than in females (37%) ($p < 0.05$). Overall, anti-HBc reactivity was lowest in the 4-5 year age group but showed no overall increase with age ($p > 0.05$ by chi-square test for trend). Mayan children showed the highest rates of infection (76%), followed by the Mestizo (50%), Garifuna (37%), and Creole (25%).

Rates were highest at rural primary schools A and B, attended predominantly by Mayan and Mestizo children respectively. At these schools, 78% and 65% of children under age nine were positive for anti-HBc and prevalence of anti-HBc showed no increase with increasing age (Table 3). In contrast, at the urban primary school attended predominantly by Garifuna and Creole children, only 10% of children under age nine were reactive for anti-HBc and infection rates did increase with increasing age ($p < 0.05$ by chi-square test for trend). Anti-HBc was found in 36% and 42% of students at high schools 1 and 2 respectively, and this reactivity showed no statistically significant increase with age. Forty of the 254 students (16%) who tested positive for anti-HBc had a history of jaundice, but this variable was not predictive of HBV infection ($p > 0.05$).

Malaria and hematocrit results

Malaria parasites (3 *P. vivax* and 2 *P. falciparum*) were detected in 5 children (0.8%), all living in rural communities and attending one of the rural primary schools. The point prevalence of malaria in the two rural primary schools was 3%. Likewise, children from the two rural primary schools were found to have lower mean hematocrit levels than those children from the urban primary school (Figure 1). Approximately 10% of children at the two rural primary schools

had hematocrit levels <33% compared to 1.7 % at the urban primary school ($p < 0.05$). Mean hematocrit levels at the two high schools were comparatively higher, as would be expected since hematocrit levels normally increase with age until puberty. Two students (1.6%) at high school 1 and no student at high school 2 had hematocrit levels <33%.

Prevaccination Serologic Testing

Based on our actual costs of serologic screening (\$7.00/child tested), and a conservative estimation of vaccine and administration costs (\$23.50/child for three doses), vaccine program savings of 37%-49% would be realized in the rural primary schools by screening and immunizing only anti-HBc negative children (Table 4). In fact, the urban primary school with anti-HBc prevalence of 22% would be the only school of those studied where mass vaccination would be more cost effective than pre-immunization screening. In our study population, pre-immunization screening would result in vaccine program cost savings when anti-HBc prevalence exceeds 30%.

DISCUSSION

This study indicates that hepatitis B infection is common in school-aged children in the Stann Creek district of Belize. Evidence of HBV infection was found in 43% of students studied and nearly 8% were positive for HBsAg indicating acute or ongoing infection. Results of this and prior studies suggest that infection rates are non-uniform, with differences observed in age, ethnicity and location. These differences in prevalence have important ramifications in terms of vaccination strategy including prevaccination screening for infection. In addition, we observed

marked differences in rates of anemia and malaria which suggest additional intervention in these populations.

The highest prevalence of HBV was found in populations living in select rural communities. One of the two rural primary schools sampled is located in a Mayan village, and the other serves a predominantly Mestizo population of immigrants from neighboring Central American countries. Unlike the other areas sampled, few residents of these communities have electricity or running water. These schools are in close proximity to a banana plantation where a 1987 investigation of hepatitis found 77% of workers infected with HBV.⁸ These schools were chosen for inclusion in the study because they were known high risk sites. Therefore, results may not be representative of hepatitis exposure in all rural Stann Creek villages. Even so, the high prevalence of hepatitis B among these Maya and Mestizo students was surprising. In previous studies of adult Maya and Mestizo in the Belize Defense Force, the prevalence of anti-HBc was only 9% and 5% respectfully.² However, most of those Maya and Mestizo adults came from districts other than Stann Creek.

Rates of infection among the predominantly Garifuna and Creole children (24% and 25%) in the urban primary school are lower than infection rates found in prior studies of adults (56% of Garifuna and 30% of Creole) residing in this area.^{2,3} This result is concordant with the association between increasing age and hepatitis B demonstrated in previous studies. At the urban primary school and at high school 1 (both located in the same city), the prevalence of hepatitis B increased with increasing age, though this was significant only in the primary school. The prevalence of hepatitis B did not increase with age among students in the two rural schools; 71%-80% of children 6-8 years of age were already infected. Similarly, rates of infection among the high

school students overall showed no increase with age, unlike some populations in which prevalence of HBV increases at sexual maturity.¹⁰ However, because ethnicity, school and location were closely linked confounding variables, the individual contribution of each of these variables to the marked variation in HBV endemicity could not be determined.

The modes of transmission of HBV in our study population are yet to be determined, and the 79% and 67% rates among students at the two rural schools call for additional investigation. However, mother-to-child transmission such as that seen in the Far East appears to be an uncommon mode of transmission among Garifuna and Creole. A 1993 seroprevalence study of pregnant women attending prenatal clinics in Belize suggests that children rarely contract HBV at the time of birth through HBsAg positive mothers (author's unpublished data). In that study, only one of 92 pregnant women residing in Stann Creek district was reactive for HBsAg. However, HBV among pregnant women in the Mayan and immigrant communities has not been adequately studied. Nevertheless, four of seven pregnant women in the Mayan community of primary school A had anti-HBc, but none were reactive for HBsAg. Predominant transmission in this population may be sibling-to-sibling, similar to mechanisms described in Africa,¹¹ though other (as yet undefined) modes of transmission may also contribute.

While greater than 90% of older children and adults who acutely develop hepatitis B will clear the infection and develop life long immunity, infections acquired during infancy and early childhood commonly become persistent.¹² An estimated 25% of children infected between the ages of one and five, and 70% to 90% of infected infants become chronic carriers of the disease.¹³ However, in the rural Mayan population we studied, early childhood infections do not seem to convey this same high risk of chronic infection. Despite high HBV infection rates (78%) prior to

age nine, only 10.5% of the 4-8 year old Mayan children with anti-HBc had indication of either active infection or chronic carrier state as evidenced by the presence of HBsAg. This lower than expected rate of HBsAg among the Maya suggests that potential differences in exposure, viral strain or immunologic response may play an important role in the natural history of HBV infection.^{13,14}

Since a majority of children in these rural communities contract HBV at pre-school ages, immunization would be most effectively given as part of an infant immunization program. By integrating the hepatitis B vaccine into the routine infant immunization program, vaccine administration, transportation, and cold-chain storage costs could be reduced substantially. Nevertheless, while the high rates of HBV infection in Stann Creek could, over time, be best controlled through infant immunization, a school based vaccination program could accelerate control and extend this protection to those (anti-HBc negative) children still at risk.⁵ In four of the five schools we studied, this immunization of school-aged children would result in vaccine program cost savings if preceded by HBV seroprevalence screening.

As outlined by the World Health Organization, vaccination of all infants in highly endemic areas is imperative. In areas of intermediate endemicity, newborns, high risk groups and members of infected households must be included in the eradication program. In areas of low endemicity such as the northern districts of Belize, screening of pregnant women to identify infants to be immunized, along with high risk groups such as health care workers must be undertaken.^{15,16} This study provides a rational basis for the implementation of a hepatitis B immunization program in the Stann Creek district of Belize, an area of high HBV endemicity. Still unanswered, however,

are questions concerning the modes of HBV transmission and determinants of disease chronicity in this region.

Table 1 Proportion of ethnic groups in Stann Creek District compared to the study population		
	% of population	% of students sampled
Garifuna	36.2	29.4
Creole	25.1	24
Mestizo	23.7	15.7
Maya	8	22.7
Other	7	8.2

Table 2
Hepatitis B prevalence by selected demographic characteristics

	Number tested	% Anti-HBc reactive	% HBsAg reactive
Total study population	587	43.3	7.7
Sex			
Male	253	52	8.7
Female	334	37	6.9
Age group (years)			
4-5	33	27	3
6-8	96	48	12
9-11	121	45	7
12-14	111	49	6
15-18	180	39	6
19-22	46	46	13
Ethnicity			
Mayan	133	76	9
Mestizo	92	50	11
Garifuna	172	37	9
Creole	141	25	4
Other	48	19	2
School			
Primary School A	95	79	6
Primary School B	55	67	20
Urban Primary	174	22	6
High School 1	126	36	7
High School 2	137	42	6

Table 3
Number of students studied (and % with anti-HBc) according to age and school

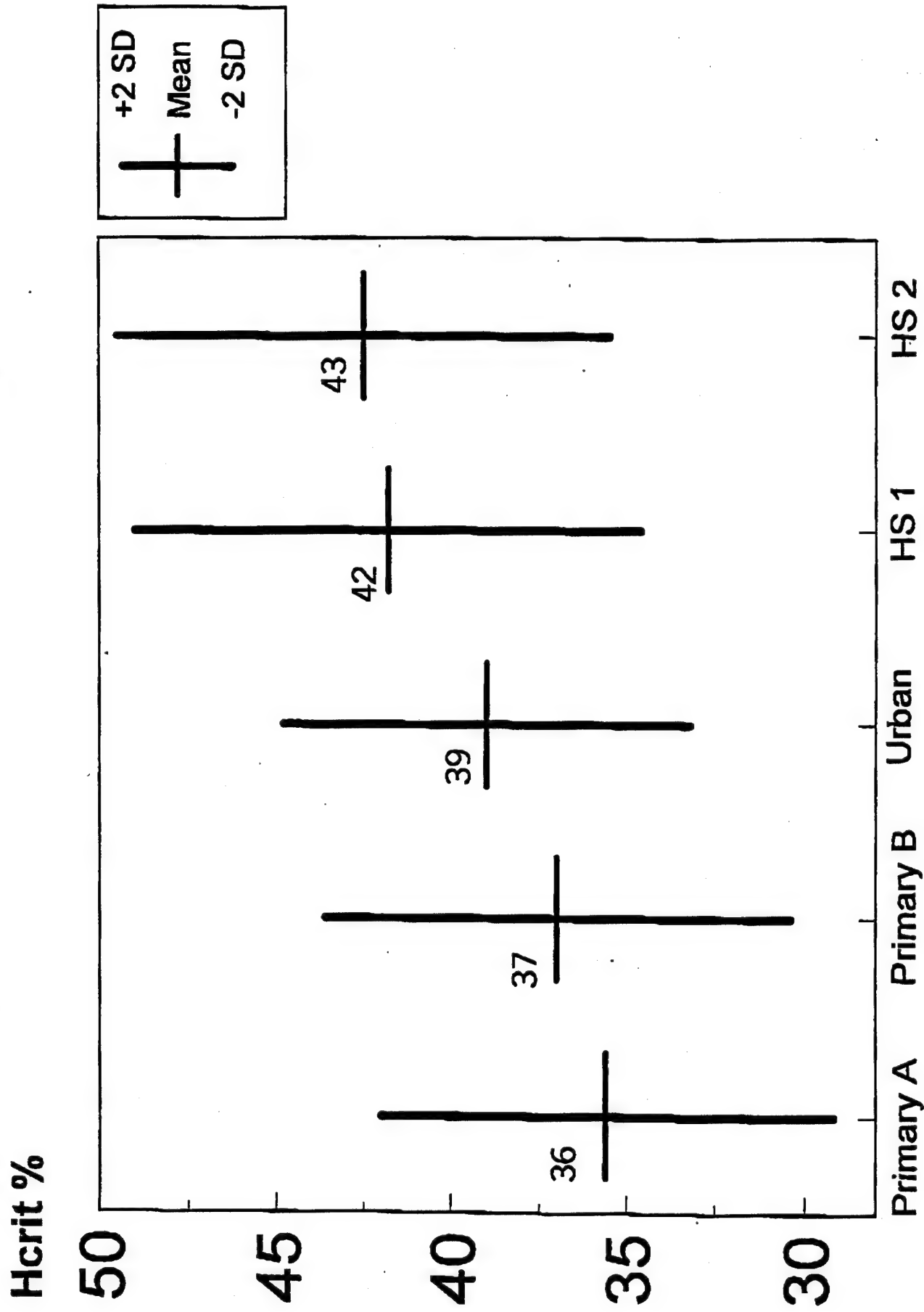
Age group (years)	Primary School A	Primary School B	Urban Primary	High School 1	High School 2
4-5	9 (67)	3 (33)	21 (10)		
6-8	40 (80)	14 (71)	42 (10)		
9-11	32 (72)	27 (59)	61 (25)		1 (0)
12-14	14 (100)	11 (91)	45 (40)	5 (20)	36 (31)
15-18				86 (33)	89 (47)
19-22				35 (46)	11 (45)

Table 4
Cost /100 students to screen and vaccinate only persons susceptible to Hepatitis B
compared to mass vaccination

School	Cost/person to screen & vaccinate	Cost/person to vaccinate all	Percent savings
Rural primary A	\$11.93	\$23.49	49%
Rural Primary B	\$14.75	\$23.49	37%
Urban Primary	\$25.32	\$23.49	-7%
High School 1	\$22.03	\$23.49	6%
High School 2	\$20.62	\$23.49	12%

Figure 1

Spun hematocrit levels by school



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ABSTRACT

Pesticide avoidance behavior in Anopheles albimanus, a malaria vector in Central and South America

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The biological effects of permethrin, deltamethrin and DDT on two laboratory colonies (Santa Tecla colony from El Salvador and El Semillero colony from Guatemala) and two field populations (Toledo and Corozal, Belize) of Anopheles albimanus were characterized by behavioral responses and susceptibility diagnostic tests. The susceptible laboratory colony (Santa Tecla) has been maintained at least 20 years and is susceptible to all three insecticides. The colony from Guatemala was recently colonized and exhibited resistance to permethrin, deltamethrin, and DDT. The third population ("wild-caught") was from southern Belize and demonstrated resistance to DDT only. The fourth population ("wild-caught") was from the northern Belize and was susceptible to all three chemicals. Each behavioral study compared escape responses of each test population from a chamber affording direct contact with insecticide-treated surfaces or from a chamber that excluded direct contact with treated surfaces. Two control chambers (one with and one without direct contact with surfaces) treated with the carrier (Risella Oil) only were included in each study. Test chambers affording direct contact with treated surfaces showed a very dramatic escape response of Guatemalan and Belizean mosquitoes for all three chemicals. Numbers of females escaping from chambers without direct contact with treated surfaces were significantly greater than number escaping from control chambers ($P < 0.05$) but less than

numbers escaping from chambers affording direct contact with insecticide. In marked contrast, few females from the susceptible laboratory colony escaped from test chambers, regardless of insecticide used. Isozyme analysis suggested that the susceptible laboratory colony has lost some genetic heterogeneity compared to field-caught and recently colonized populations. Comparison of esterase activity between pyrethroid susceptible and resistant populations was performed using a microtitre plate assay. Comparatively high activity of esterase was found in resistant populations compared to the susceptible populations. This suggests that the presence of this elevated esterase in the Guatemalan colony may limit the use of pyrethroids in malaria control. We conclude that behavioral responses of malaria vectors are an important aspect in the insecticide-malaria control equation and that this aspect warrants further study in the laboratory and the field.

CHAPTER 5

SUMMARY DISCUSSION AND CONCLUSION

TABLE 1. Presence of physiological resistance and behavioral avoidance in An. albimanus.

	Physiological resistance			Behavioral avoidance		
	DDT	Permethrin	Deltamethrin	DDT	Permethrin	Deltamethrin
Santa Tecla	-	-	-	-	-	-
El Semillero	+	+	+	+	+	+
Toledo	+	-	-	+	+	+
Corozal	-	-	-	+	+	+

-; absence +; presence

Isozyme analyses indicated that there were no real genetic differences among three laboratory colonies (Santa Tecla, El Semillero, and southern Mexico) and three field populations from Toledo, Cayo, and Corozal Districts in Belize, suggesting an absence of cryptic species. Our evidence that the geographic populations of An. albimanus represent a single species is in agreement with other studies that used ribosomal DNA (rDNA) analysis (Beach et al., 1989a), cytogenetic analysis (Narang et al., 1991), isozyme analysis (Narang et al., 1991), hybridization crosses (Narang et al., 1991), egg morphology (Rodriguez et al., 1992), and genetically acquired host preferences (Arredondo-Jimenez et al., 1992). Among 31 enzyme systems tested, 24 showed consistent activity and 35 loci were scorable. Higher genetic variability was found in young colonies (e.g., El Semillero) from Guatemala and Mexico and three field populations from Belize compared to the old colony from El Salvador. Of six populations examined, the old (i.e., Santa Tecla) colony showed a drastic reduction in genetic variability by displaying low allelic polymorphism. The three field populations from Belize had higher genetic variability than the laboratory colonies from Guatemala and Mexico. The Santa Tecla colony was the more extreme case,

being physiologically susceptible to all three insecticides and exhibiting no escape behavior to avoid the insecticides. In another study, Robert *et al.* (1991) reported that Santa Tecla females had a very low repellency response to N, N-diethyl-3-methyl-benzamide (deet) compared to females of other species. Brown (1958) found that a 20-year old laboratory colony of *An. albimanus* had a two times lower excitability response to DDT than wild populations of the same species. Collectively, these observations indicate that laboratory colonies can quickly lose the behavioral capacity to respond to insecticides.

Genetic isozyme expression was compared between pyrethroid resistant and susceptible populations. Of 35 loci, one locus of esterase and two loci of leucine-amino-peptidase were diagnostic of susceptible populations versus populations displaying moderate levels of pyrethroid resistance. Polyacrylamide gel electrophoresis (PAGE) was used to confirm the relative mobility of *Est-3* between susceptible and resistant populations that displayed almost similar mobility on horizontal starch gels. The increase of leucine-amino-peptidase in pyrethroid-resistant arthropods has not been reported previously. This enzyme may be involved in the metabolic functions of pyrethroid resistance in arthropods. Additional work on esterase and leucine-amino-peptidase metabolic pathways of pyrethroid resistance is needed.

Pyrethroids are insecticidal esters derived from primary alcohols and are susceptible to hydrolysis by esterase (Kerkut & Gilbert, 1985). Pyrethroid-resistant *Spodoptera littoralis* (Egyptian cotton leafworm) was characterized with elevated esterase activity (Riskallah, 1983). Similarly, deltamethrin-resistant *An. albimanus* populations demonstrated elevated esterase levels in three localities of Guatemala (Beach *et al.*, 1989b). Our findings showed a four-to-seven-fold increase in esterase activity in a pyrethroid-resistant colony of *An. albimanus* compared to field populations from Belize or the susceptible Santa Tecla colony from El Salvador.

Resistance to pyrethroids seems widely distributed in *An. albimanus* populations of Guatemala. Malcolm (1988) reported deltamethrin resistance in *An. albimanus* in six

localities of Guatemala, and Beach *et al.* (1989b) reported resistance in three localities of Guatemala. Permethrin and deltamethrin have been used by the Guatemalan malaria control programs since 1986 (Bisset *et al.*, 1991) and the pyrethroids may act as selecting agents. Furthermore, pyrethroid resistance occurs in DDT-resistant populations as reported for an *Ae. aegypti* population from Thailand (Brealey *et al.*, 1984) and Guyana (Prasittisuk & Busvine, 1977). In addition, larval pyrethroid resistance was reproduced in a DDT-selected strain of *An. stephensi* from Pakistan (Omar *et al.*, 1980). In our study, the World Health Organization diagnostic test also indicated that the pyrethroid-resistant colony from Guatemala was resistant to DDT. Synthetic pyrethroid resistance may have arisen from previous exposure to DDT.

DDT was used for malaria control in the Toledo District for several years before 1990. Gramoxone, a DDT-like herbicide has been used for the weed control in rice fields, the *An. albimanus* habitat in Toledo District. DDT for malaria control and Gramoxone for weed control may be responsible for the DDT resistance in *An. albimanus* populations from Toledo District. No resistance has been detected in the Corozal population despite DDT being used in the northern Belize for malaria control. In spite of a heavy use of DDT in the past and recent use of pyrethroids in Guatemala, only a comparatively moderate level of physiological resistance, based on estimates of percent mortality (Table 2), has developed in Guatemalan colony of *An. albimanus*. This raises the possibility that behavioral avoidance of insecticides in these three populations serves to ameliorate the selective pressure for resistance in *An. albimanus* of Guatemalan as seen in *An. sacharovi* in Greece (de Zulueta, 1959).

TABLE 2. Percent mortality (n=3) of adult An. albimanus at the single diagnostic dosage (World Health Organization susceptibility test, 1975).

Population or colony	DDT (4.00%)	Permethrin (0.25%)	Deltamethrin (0.025%)
Santa Tecla	100	100	100
El Semillero	45	50	50
Toledo	65	100	100
Corozal	100	100	100

Muirhead-Thomson (1960) suggested the term "protective avoidance" over "behaviouristic resistance". Protective avoidance can be defined as the natural ability of mosquitoes to avoid the irritation of insecticide without previous exposure to insecticide. Behaviouristic resistance referred to a population gradually developing the ability to avoid the insecticide over time. The term "resistance" can be defined as "the developed ability in the strain of insects to tolerate doses of insecticides which prove lethal to a normal population of the same species" (World Health Organization, 1957). Therefore, the development of resistance is dependent on genetic variability already present in a population (Oppenoorth, 1984). To avoid an ambiguous interpretation, the term "behavioral avoidance" is preferred since behavioral changes by selective pressure from insecticide exposure in nature has not been adequately documented (Muirhead-Thomson, 1960).

Mechanisms of behavioral avoidance to insecticides remain a mystery since most studies have concentrated on physiological resistance mechanisms. The investigation described in this report was designed to determine if an association exists between physiological resistance and behavioral avoidance of insecticides in Anopheles albimanus

populations. Based on the results presented in Table 1, there appears to be no association between physiological resistance and behavioral avoidance in our test populations of An. albimanus. Strong behavioral avoidance was seen in three populations of An. albimanus; some of which were physiologically resistant to DDT and/or pyrethroids, i.e., no relationship between physiological resistance and behavioral avoidance was detected. Based on percent mortality at diagnostic doses of insecticides (Table 2), the El Semillero colony showed resistance to deltamethrin, permethrin, and DDT; whereas, the Belizean field population from Toledo exhibited resistance to DDT, but no pyrethroid resistance. The Santa Tecla colony and field populations from Corozal, Belize were susceptible to all insecticides. A lower degree of physiological resistance was found in field populations from Toledo compared to susceptibilities of the El Semillero colony, while Toledo populations demonstrated much stronger behavioral avoidance than the El Semillero colony. This indicates that the tendency of mosquitoes to leave DDT treated surfaces is not based solely on the level of physiological susceptibility. The Corozal population showed susceptibility to DDT and pronounced avoidance behavior. This again indicates that avoidance behavior is independent of physiological resistance; i.e., they are not necessarily inter-related as proposed by Lockwood et al., (1984). Among 80 cases of dipterans reported to show behavioral avoidance of pesticides, only 16 cases (20%) were known also to have physiological resistance (Lockwood et al., 1984). This suggests that no fixed or universal relationship between behavioral avoidance and physiological resistance is present in the Diptera.

Adult An. albimanus females from Toledo and Corozal Districts of Belize and from El Semillero of Guatemala demonstrated strong, unambiguous escape responses when placed in contact with DDT, permethrin or deltamethrin. Contact with insecticide produced a more immediate egress response and a much higher percentage of test individuals quickly escaping test chambers compared to non-contact trials. Higher doses caused more mosquitoes to escape than lower doses. Ree & Loong (1989) found that An. maculatus

showed an increased irritability response to increasing doses of permethrin. In our 30-minute exposure periods, dose levels appeared to have no effect on rate or patterns of escape in non-contact repellency tests. Dose levels did influence escape behavior in tests conducted for four-hour exposure periods. Mosquitoes from different geographic locations also show variability in escape rates as described by Busvine (1964).

Results from four-hour exposures (long term) to DDT, permethrin, and deltamethrin suggest that non-contact repellency is an important behavioral response in An. albimanus. In general, deltamethrin showed the most repellency, followed by permethrin and DDT. Unlike our finding with 30-minute exposures (short term) in non-contact trial, greater escape activity was seen at higher versus lower doses for all three compounds in four-hour exposures.

Many synthetic pyrethroids cause mosquitoes to escape sprayed houses (Miller, 1990; Lindsay et al., 1991). Our results demonstrated that both permethrin and deltamethrin produce strong behavioral escape responses from An. albimanus females. This is presumably due to the irritation caused by the chemical when mosquitoes come in contact with treated surfaces. Based on available information, it is difficult to determine how avoidance behavior originates. Avoidance behavior might be associated with selective pressure from previous exposure to synthetic pyrethroids or closely related insecticides. However, the more probable explanation is that avoidance behavior is an innate response to related classes of natural chemicals. The responses of field populations from Corozal and Toledo Districts suggest innate behavior since both populations have no history of prior exposure to synthetic pyrethroids through agricultural or public health use.

Both repellency and irritancy to DDT, permethrin, and deltamethrin were observed in An. albimanus populations. Although, true DDT repellency was documented for An. darlingi in Brazil (Roberts & Alecrim, 1991), repellency played a secondary role in the escape responses of An. albimanus females in our study. We found that DDT, permethrin, and deltamethrin have a strong irritant action and a limited repellent action on

An. albimanus females, causing them to escape exposure chambers, and to generally survive insecticide exposure. Synthetic pyrethroids had slightly stronger irritancy and repellency effects than DDT did on An. albimanus. Both repellency and irritancy effects may provide protection against indoor man-vector contact. These findings are in agreement with Roberts & Alecrim (1991) who reported DDT demonstrates a strong repellent action. A repellent action that exerts an area effect would theoretically provide significant protection from indoor transmission of malaria. However, others propose that the irritant properties of permethrin and deltamethrin in treated huts have an unsatisfactory impact on malaria vectors leading to escape and survival of mosquitoes (Rishikesh et al., 1978). This reasoning led to the termination of DDT use in many countries in Soviet Central Asia, Asia, and South Africa due to high irritancy (Bondareva et al., 1986; Sharp et al., 1990). However, malaria transmission decreases when the man-vector contact rate is reduced, and the absolute mosquito population size may not be the most important component. Additional field work is needed to study the impact of excito-repellency of insecticides and mosquito density in relation to malaria transmission.

While no method of analysis of the excito-repellency test has been fully accepted (Roberts et al., 1984), survival analysis was developed as a method of choice for excito-repellency data in this study. This analysis is advantageous by providing probability of escape over time, comparisons of response rates and patterns with exposure time. In this analysis, escaped mosquitoes are considered "dead", while the non-escaped mosquitoes are judged as "survive". A log-rank method was used for comparing the escape response behavior, and a p-value was calculated to show the differences between survival curves. This analysis was designed to minimize the loss of valuable information that might have arisen by using graphical comparisons.

Although a portable excito-repellency test chamber provided capacity to test insecticides for behavioral responses rapidly and accurately, a standard metal collapsible, excito-repellency test chamber should be developed to avoid transportation problems for the

field use. In our study, the test chambers are inexpensive and were designed for both contact and non-contact trials.

In conclusion, behavioral responses of malaria vectors to insecticides are important components of the insecticide-malaria control equation. These responses are invariably overlooked in vector control program. Furthermore, development of resistance in malaria vectors to DDT have never been reported in some countries despite its long term use (Roberts & Andre, 1994) suggesting behavioral avoidance as the principal mechanism of control, not toxicity. More field research is needed on the behavioral responses of vector populations from different geographical locations to various insecticides. As seen from the old (Santa Tecla) colony results, we recommend that laboratory colonies should be excluded or used cautiously when conducting insecticide susceptibility and behavioral studies.

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THE PREVALENCE OF HEPATITIS A, B AND C INFECTION AMONG DIFFERENT ETHNIC GROUPS IN BELIZE

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Abstract. Little is known about the prevalence of infection with hepatitis viruses in Belize, Central America. We conducted a serologic survey among members of the Belize Defence Force (BDF), which is composed of the five major ethnic groups in Belize, to estimate prevalence rates of hepatitis A, B, and C among military-aged men and women in Belize. Of approximately 600 men and women in the BDF, 492 (82%) completed a questionnaire and blood collection. Antibody to hepatitis A was found in 94%, with similar rates by age, sex, rank, and ethnicity. Antibody to hepatitis B core antigen (anti-HBc) was found in 31%. Rates of anti-HBc varied significantly among the ethnic groups with the lowest rates in Mestizo (5%) and Mayan Indians (9%), and significantly higher rates among Creoles (30%) and Garifuna (56%). Rates increased with increasing age from 28% in those 18-24 years old to 35% in those ≥ 35 years old ($P = 0.07$, by chi-square test for trend). Hepatitis B surface antigen was found in 21 (4%) overall. Antibody to hepatitis C was found in two (0.4%). In this young healthy population, exposure to hepatitis A before the age of 18 is almost universal, while exposure to hepatitis B is related to age and ethnic origin.

There have been no reports of the prevalence of viral hepatitis for the Central American country of Belize. The only serologic studies of hepatitis virus markers in Belize are from a recent outbreak investigation in a community of immigrant banana farm workers.¹ The following study was conducted during the summer of 1992 to determine the prevalence of hepatitis A virus (HAV), hepatitis B virus (HBV), and hepatitis C virus (HCV) infection in a Belizean population, the Belize Defence Force (BDF).

The BDF is a light-infantry battalion, supported by air and maritime wings. Formed in 1978, the combined strength of active duty personnel is approximately 600. Since members of the BDF are recruited from five diverse ethnic groups from all six districts of the country, the survey was done to obtain information about the prevalence of hepatitis A, B, and C in these ethnic groups, and to determine the need for preventive intervention in this military organization.

MATERIALS AND METHODS

The study was approved by a Human Use Review Committee of the Ministry of Health of Belize, the Defence Board in Belize, and the Human Use Review Committee at the Uniformed

Services University of the Health Sciences. All available personnel of the BDF were invited to participate in the study. After obtaining informed consent from each volunteer, questionnaire data (which included identifying demographic, behavioral, and medical history information) was collected by interviewers. A 12-ml venous blood sample was then collected and labeled with a unique identification number. Only the principal investigator had access to the code linking the number to the volunteer's questionnaire.

Serum samples were then tested by enzyme-linked immunosorbent assays (ELISAs) for antibody to HAV (anti-HAV) (Hepanostika® anti-HAV), hepatitis B surface antigen (HBsAg) (NML® ELISA HBsAg), and antibody to HBV core antigen (anti-HBc) (Hepanostika® ANTI-CORE) (all from Organon Teknika Corporation, Durham, NC). Testing for anti-HCV was done with an anti-HCV second generation enzyme immunoassay (Abbott Diagnostics, Abbott Park, IL). All tests with positive results were repeated at least twice for confirmation.

RESULTS

Study population

There were 519 subjects, which represented 87% of eligible BDF members, entered into the

TABLE 1

*Proportion of ethnic groups in the population of Belize and the Belize Defence Force (BDF)**

	% of population	% of BDF
Creole	29.8	33.0
Garifuna	6.6	33.0
Mestizo	43.6	18.3
Mopan Maya	3.7	9.7
Ketchi Maya	4.3	1.5
East Indian	3.5	1.6
Other	8.5	3.0

* The total population of Belize in 1991 was 184,722 and the BDF had 600 members.

study. Questionnaire data was available on 492 (95%) of these. These 492 subjects comprise the study population. Of the 472 males and 20 females, there were 455 (92%) enlisted personnel, 35 officers (80% of all officers of the BDF) and two civilians. The age range was 18–44 years, with the mean age of the enlisted personnel and officers being 25 years and 29.6 years, respectively.

The racial/ethnic composition of the study population of the BDF in comparison with the total population of the country of Belize is shown in Table 1. While the Creole component of the BDF comprised a similar proportion to that of the general population of Belize, the Garifuna group comprised a greater proportion and the Mestizo a lesser proportion in the BDF than in the general population. This survey was also not representative of the indigenous people of Belize (the Mopan and Ketchi Maya). Whereas the Mopan Maya predominantly live in three districts in the country, almost all (92%) of those in the survey were from the southern district of Toledo, and 50% were from a single village. The Mopan Maya were over-represented in the BDF while the six Ketchi Maya were under-represented.

Hepatitis A prevalence

Almost all of the study participants had evidence of prior HAV infection (94% were reactive to anti-HAV). There was no difference in prevalence of anti-HAV by sex, ethnic group, officers versus enlisted personnel, or age group (Table 2). Only 18 (4%) of the 464 with anti-HAV reported a history of jaundice; 10 of these 18 also had serologic evidence of previous infection with HBV.

TABLE 2

*Hepatitis A (HAV) and B seroprevalence in Belize Defence Force personnel, by selected demographic characteristics**

	No. tested	% anti-HAV reactive	% anti-HBc reactive
Total study population	492	94	33
Male	472	94	31
Female	20	100	45
Rank			
Enlisted	455	95	32
Officer	35	91	29
Civilian	2	100	0
Ethnicity			
Garifuna	163	98	56
Creole	162	90	30
Mestizo	90	96	5
Maya Mopan	48	100	10
Ketchi Maya	6	83	0
East Indian	8	88	13
Other	15	87	33
Age group (years)			
18–24	247	95	28
25–29	130	92	32
30–34	75	95	40
≥35	40	98	35

* Anti-HBc = antibody to hepatitis B virus core antigen.

Hepatitis B prevalence

One hundred twenty-one subjects (33%) were reactive for anti-HBc and 21 (4%) had HBsAg. The combined Ketchi and Mopan Mayan component of the BDF had a 9% prevalence for anti-HBc. The Mestizo (mixed Mayan and Spanish) ethnic group had a 5% prevalence for anti-HBc. For the Creole group (of mixed African ancestry), the prevalence of anti-HBc (30%) was significantly higher than in the Mestizo or Maya ($P < 0.001$). Anti-HBc was found most commonly in the Garifuna component of the BDF (56%), a significantly higher percentage than for the Creole group ($P < 0.001$) (Table 2). In addition, a higher proportion of the Garifuna with anti-HBc were positive for HBsAg (19 [21%] of 91) compared with two (3%) of 49 of the anti-HBc-positive Creole population ($P < 0.01$).

The prevalence of anti-HBc generally increased with age, from 28% in subjects 18–24 years old to 40% in those 30–34 years old, but the prevalence was only 35% in those > 35 years ($P = 0.07$, by chi-square test for linear trend). Compared with the prevalence of anti-HBc in Creoles, which showed a general increase with

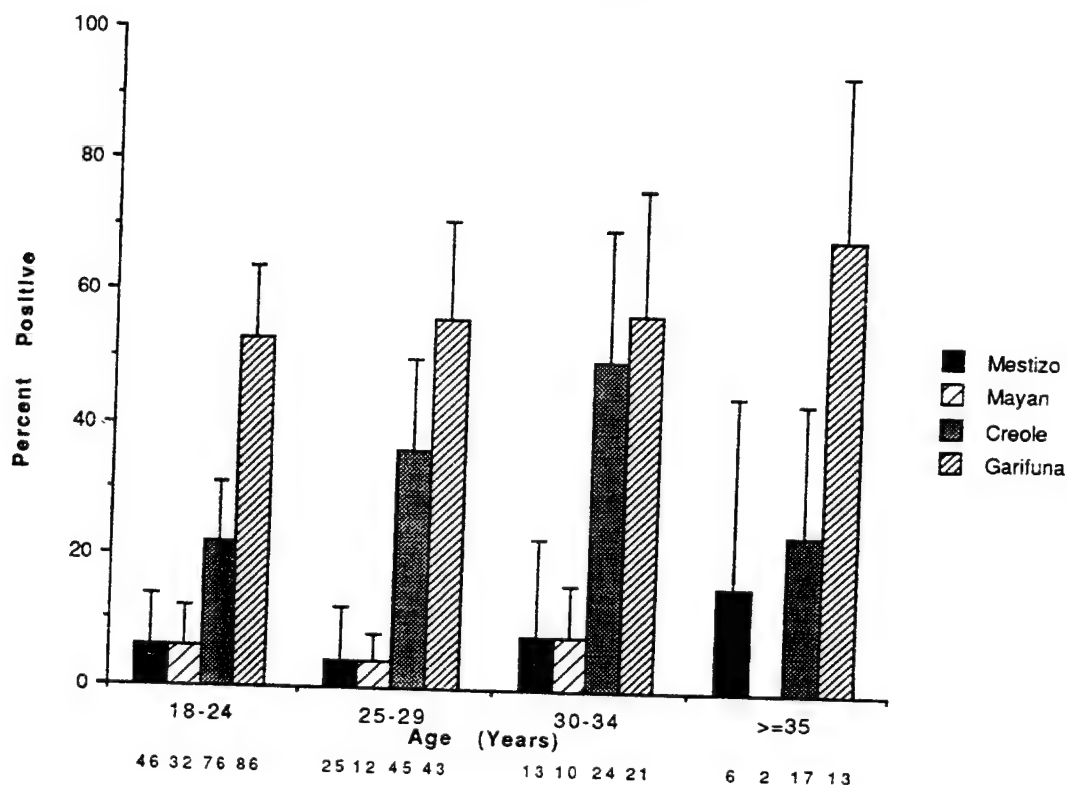


FIGURE 1. Proportion of the Belize Defence Force with antibodies to hepatitis B virus core antigen (anti-HBc), by age and ethnic groups. Bars indicate the upper 95% confidence interval. Values below the ages indicate the number studied in each group.

age from 22% in subjects 18–24 years old to 50% in subjects 30–34 years old ($P = 0.15$, by chi-square test for linear trend), the prevalence of anti-HBc in the Garifuna component of the BDF remained stable (53–57%) in those 18–34 years old (Figure 1). The prevalence of anti-HBc was not significantly different among the seven enlisted ranks ($P = 0.2$) and did not significantly correlate with years of service in the BDF ($P = 0.09$).

Risk factors known to be related to HBV transmission and included in the study are shown in Table 3. Anti-HBc was more commonly found in those with a history of pierced earlobe(s) (38% versus 30%; $P = 0.07$). Those with both pierced ears and tattoos had a higher prevalence of anti-HBc (40%) compared with those who had neither (31%; $P = 0.18$). However, there were no statistically significant associations between the risk factors listed and anti-HBc reactivity. Because of issues of confidentiality, information about

sexual practices potentially related to HBV transmission was not included in the questionnaire.

Hepatitis C prevalence

Only two (0.4%) of 492 subjects had repeatedly reactive anti-HCV. One of the cases was also reactive for anti-HBc. Neither subject gave a history of hepatitis, receipt of blood transfusions, nor ear piercing. One had a tattoo.

DISCUSSION

In this study, there was a high prevalence of antibody to HAV (94%), with a low rate of a history of jaundice, suggesting asymptomatic infections acquired early in life. This pattern of HAV seroreactivity is consistent with findings in other economically developing countries where infection occurs primarily in young children, and

almost all have antibody to HAV by the age of 10 years.²

Evidence of HBV infection was found in 33% of the BDF personnel studied and 4% were positive for HBsAg. In the previously mentioned study of an outbreak investigation of hepatitis in rural Belize, evidence of HBV infection was found in 77% of workers on a banana farm and 18% were positive for HBsAg.¹ In that study, the sample population consisted mainly of refugees from the neighboring Central American Republics of El Salvador and Guatemala. No individuals of refugee status were included in our study population.

The results suggest that HBV infection is concentrated in a small segment of the population of Belize. The Garifuna are descendants of African slaves and Carib Indians who settled in the Bay Islands of Honduras and in coastal settlements of the southern districts of Stann Creek and Toledo in Belize about 1800. The prevalence of HBV in this ethnic group in the BDF was several times that of the combined indigenous Maya component or the Mestizo group, and almost twice that of the Creole population.

The relatively stable prevalence across all age groups for the Garifuna component of the BDF may indicate perinatal and/or pre-adolescent (horizontal) transmission of HBV such as that which occurs among nations of Southeast Asia and the Pacific basin, and in some countries in Africa and South America.³⁻⁵ The fact that a relatively large proportion (21%) of the Garifuna with anti-HBc had HBsAg also suggests that infection may be occurring at an early age with resultant chronic carriage of HBsAg.

In contrast, the prevalence of anti-HBc in the Creole population increases markedly across the various age groups, rising from 22% in those 18-24 years old to 50% in those 30-34 years old, suggesting infection acquired in adulthood. This may indicate modes of transmission similar to those in North America and Western Europe, in which HBV infection is spread mainly through sexual contact and/or intravenous drug abuse.^{3, 6, 7} It is our observation that intravenous drug abuse is uncommon in the BDF. The human immunodeficiency virus, which shares modes of transmission with HBV, has not been found in more than four years of mandatory screening of BDF recruits including those in the present study. Thus, modes of transmission of HBV in Creoles requires additional study.

TABLE 3
Potential risk factors for hepatitis B in the study population*

	No. anti-HBc reactive/no. tested	% reactive	Odds ratio	P
Blood transfusions				
Yes	5/15	33		
No	151/477	32	0.8	0.9
History of hepatitis				
Yes	4/10	40		
No	152/482	32	1.4	0.6
Tatoos				
Yes	91/286	32		
No	65/206	32	1.0	0.95
Ears pierced				
Yes	45/117	38		
No	111/375	30	1.5	0.07

* Anti-HBc = antibody to hepatitis B virus core antigen.

Other populations with Carib Indian or West African ancestry have previously been noted to have high rates of hepatitis B. An investigation of an epidemic of severe hepatitis that occurred among the Yucpa Indians of Venezuela, who are of Carib Indian ancestry,⁸ revealed a high prevalence of anti-HBc (94%) and HBsAg (64%). Evidence of infection with the delta viral agent (HDV) was found in 86% of those with HBsAg.⁹ Hepatitis B is endemic in West Africa, the origin of the majority of slaves who were transported to the Caribbean. In Senegal, 80% of the population is infected with HBV by seven years of age.⁵

Another interesting observation is the low prevalence of anti-HBc (9%) in the indigenous people of Mayan descent in the BDF. This contrasts with high prevalence in other indigenous populations of the Americas (Alaskan Eskimos, American Indians, and Guatemalan refugee camps in southeast Chiapas, Mexico).^{10, 11}

The potential risk for infection with HDV, as occurred in the Yucpa Indians of Venezuela and the study population in Chiapas, Mexico,^{9, 11} also exists for the Garifuna. This is in addition to the enhanced risk of serious sequelae associated with chronic HBV infection such as chronic active hepatitis, cirrhosis, liver failure, and primary hepatocellular carcinoma.¹² No evidence of HDV infection was found in the HBsAg carriers identified in the outbreak investigation of hepatitis in southern Belize.¹

Since the BDF sample population is restricted

to individuals ≥ 18 years of age, it is recommended that a further cross-sectional study be done of preschool and school-aged children in the Stann Creek district, where approximately half of the Garifuna population of Belize lives. If most infections occur during infancy or childhood, an aggressive program of immunization aimed at young children could result in a substantial decrease in the prevalence of HBV in the Garifuna community, as has been seen in The Gambia.⁵ In that country, vaccination reduced the prevalence of HBV infection in immunized children from 41% to 5% in one village and from 76% to 19% in another. A strategy for reducing transmission in soldiers would include education and vaccination.

In the interim, screening of pregnant Belizean women and blood donors is of paramount importance. Such prenatal testing for HBsAg should identify at-risk newborns who require immunoprophylaxis and vaccination for the prevention of perinatal infection as a component of a comprehensive strategy to control HBV transmission in Belize.^{13, 14}

Antibody to HCV was uncommon in this study population (0.4%). Since hepatitis C is transmitted mainly through intravenous drug use or blood transfusions, this low prevalence is consistent with the low prevalence of observed intravenous drug use in the BDF. The low prevalence further indicates that this population with a high prevalence of anti-HBc may continue to donate blood with minimal risk of transmitting hepatitis C.

Disclaimer: The opinions and assertions contained in this article are not to be considered official or to necessarily represent the views of the Uniformed Services University of the Health Sciences.

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USE OF QUESTIONNAIRE TO
STUDY MALARIA INCIDENCE IN
BELIZE, C.A.

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1. INTRODUCTION

Malaria is spread to humans by the bite of the *Anopheles* mosquito and is present in most tropical areas. Rates of Malaria have been growing world wide over the past several years. In Belize, Central America, malaria is a growing problem as well. Although the increase in active case detection is partially responsible, the rates of positive blood smears has nearly doubled from 1991 to 1993 (fig. 1). Indoor spraying as a method of malaria prevention has not been conducted in Belize for the past two and one-half years. By far, most of malarial infections in Belize are by *Plasmodia vivax* (96.5%), with *P. falciparum* and *P. malariae* accounting for the remainder of cases (Pan American Health Organization, 1989).

Malaria Epidemiological Data 1975 - 1993 Belize

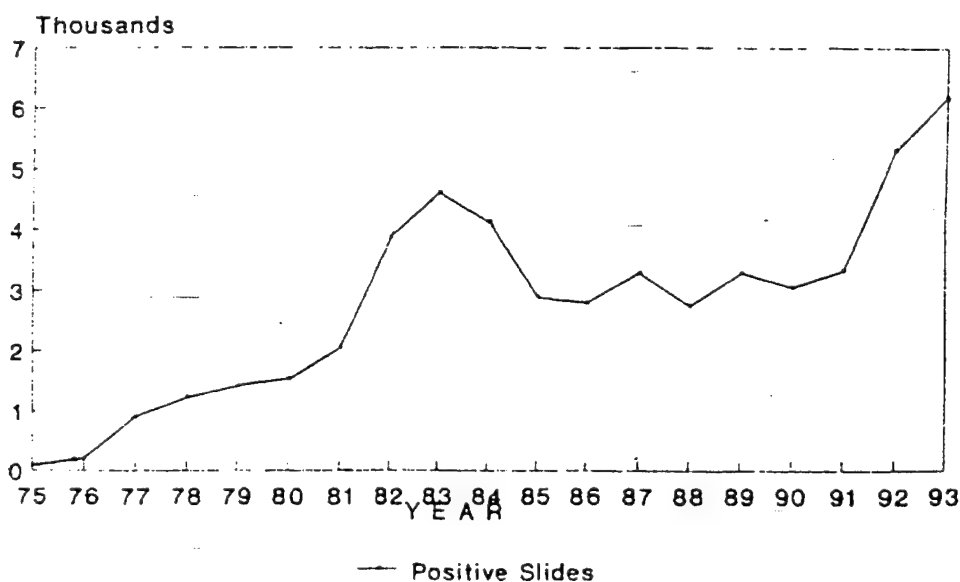


fig. 1 Malaria Rates, Belize, C.A.; Min. of Health, 1993

This research assesses the lifestyles present within the village of Red Bank, a single Mayan community in Belize. It focuses on those behaviors known to be associated with malaria acquisition such as patterns of mobility, personal protective measures, and appropriateness of most recent treatment. The primary and secondary hypothesis of this study were, respectively:

Transmission from vector to human and visa versa is occurring primarily in the Red Bank village itself.

A considerable number of malarial episodes experienced by Red Bank residents are due to recurrence as opposed to primary infections.

In determining the status of any vector-borne disease, one must consider the triad of environment, vector, and host. Similarly, the control of malaria involves a multi-focal approach of: environmental manipulation to reduce the vector source, health education, chemotherapy, and chemoprophylaxis.

Environment

Belize, approximately 4,000 square miles, is situated south of the Yucatan Peninsula, Mexico, and borders Guatemala to the west (fig. 2). The village of Red Bank lies south of Belize City in

the Stann Creek District. In 1993, Stann Creek District had the third highest malaria rate in the country (out of six) (Ministry of Health, 1993).

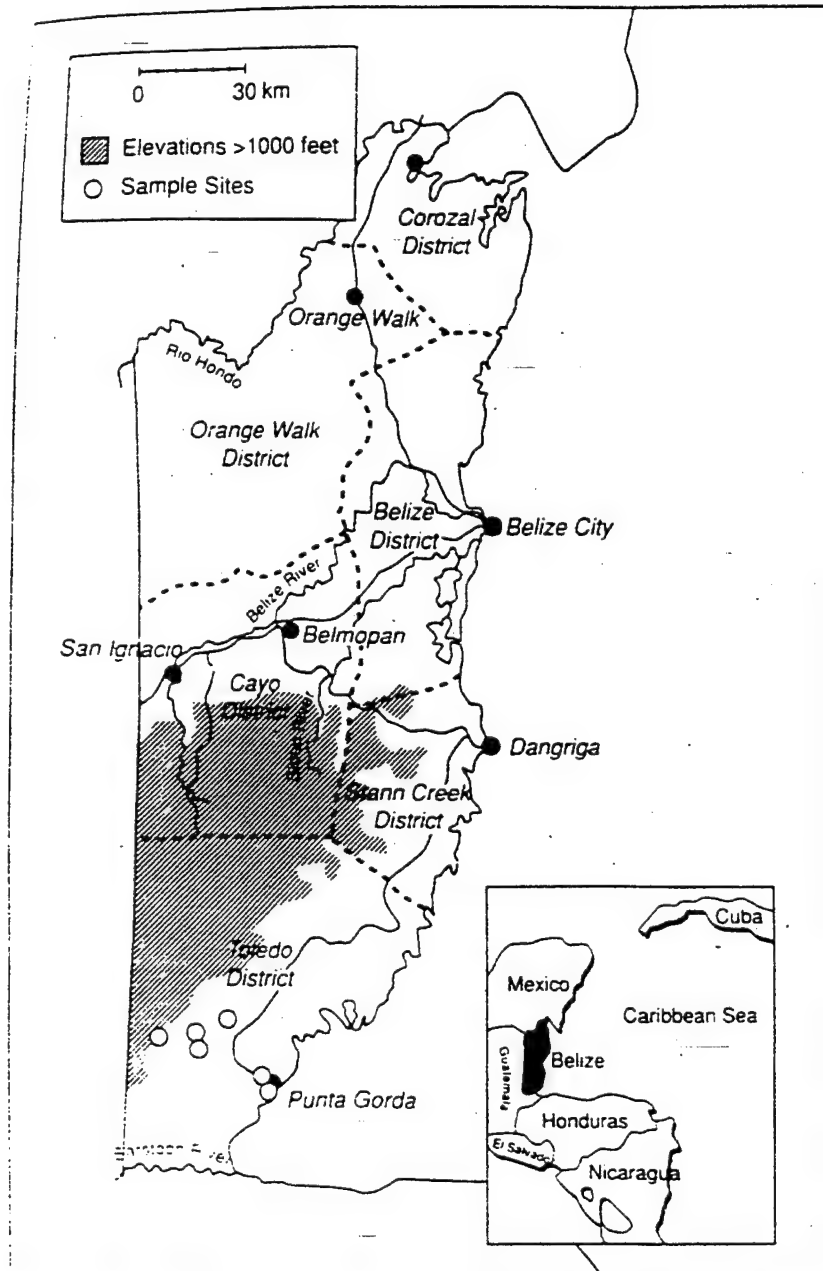


fig. 2 Map of Belize, C.A.

Red Bank is between the Caribbean coast and the Mayan mountains, 15 miles from each. The village itself is cleared but is surrounded by thick vegetation. Less than one mile from the Swasey River, the villagers live in clusters of one room thatch houses. There are a few community wells, but no sources of plumbing or electricity.

This study addresses the environmental component of the malaria problem by gathering data on how villagers interact with the environment in ways known to be associated with malaria acquisition.

Host

Approximately 205,000 people live in Belize. The ethnic groups most represented in Belize are Creole, Garifuna, East Indian, Mopan Maya, Kekchi Maya, Yucatec Maya, Mestizo, and Mennonite. Additionally, Hondurans, Guatemalans, Salvadorans, and Mexicans have migrated to Belize seeking employment, and are collectively referred to as "Ladinos". Malaria rates differ considerably between these ethnic groups. For instance, in the Stann Creek District, migrant and Mayan communities have four to 15 times the rates of surrounding Creole and Garifuna villages (fig. 3). Independence (Ind.) is a Creole township; Dangriga (Dan.) and Hopkins (Hop) are predominately Garifuna; Trio Bladden (Trio) and CowPen (CP) are migrant farm working communities; and the

the remaining villages, Maya Center, San Roman, and Red Bank, are Mayan. Again, some differences are likely due to differing case detection rates.

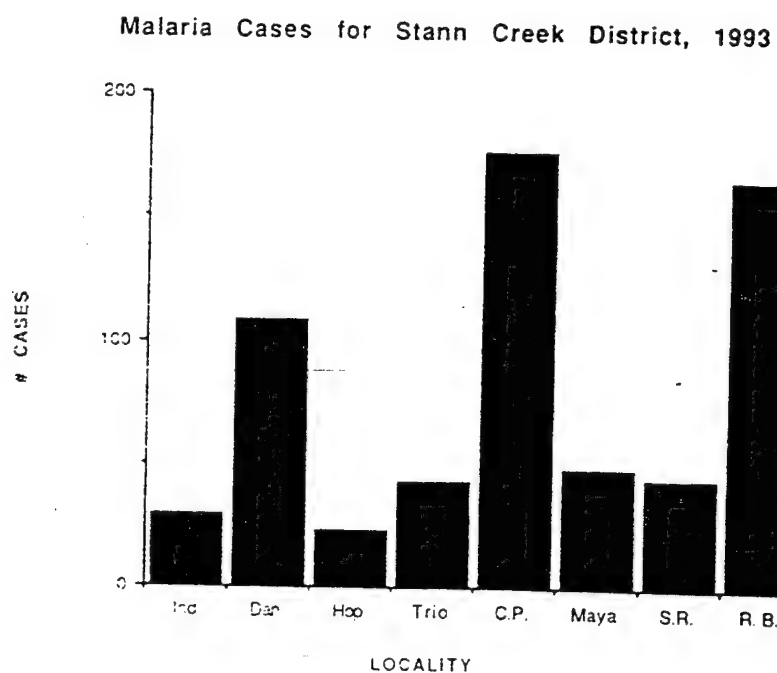
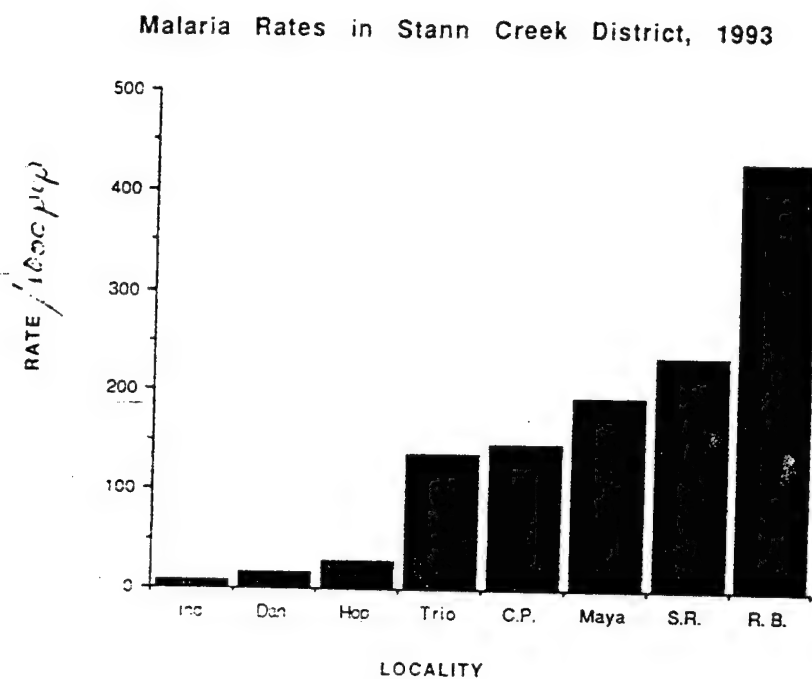


fig. 3

Red Bank, inhabited primarily by Mopan Maya Indians, is home to approximately 300 people in roughly 60 households. The Red Bank village experiences some modern influences but largely maintains its traditional culture. Nuclear families, as opposed to extended, live together with the men as head of the household. In addition, men farm the surrounding hillsides and frequently work for the owner of a nearby banana plantation. Women, on the other hand, rarely leave the immediate village and are responsible for their children and maintaining the home.

Vector

Four malaria vectors are known to exist in Belize. They are *Anopheles darlingi*, *A. albimanus*, *A. pseudopunctipennis*, and *A. vestitipennis*. Although recent entomological surveys have not been conducted in Red Bank itself, the preferred habitats of each *Anopheles* species have been determined in adjacent areas. *A. darlingi* is strongly associated with rivers and is found mainly in hot humid lowlands. *A. pseudopunctipennis* is more abundant in the foothills where it prefers the algae filled river pools in the dry season. *A. albimanus*, abundant during the wet season, is found mainly in low altitude coastal wetlands. In some areas of Belize, *A. vestitipennis* may play a role in transition of malaria but its preferred larval habitat is unknown (Rejmankova et al., 1993).

Each of these vector species is exophillic, preferring to bite outdoors. However, all will enter an open dwelling to feed on humans. As well, they follow the typical *Anopheles* pattern of feeding between the hours of dusk and dawn. This study did not attempt to determine relative abundance of vector species, biting capacities, or other entomological determinants of disease transmission. However the study does describe some aspects of human risk and perceptions regarding vectorial behavior.

II. MATERIALS AND METHODS

In this retrospective cohort study, data were collected by the administration of a questionnaire (Appendix A). The village of Red Bank was chosen as the study site because of its high incidence of malaria (50%) and its ethnic purity. A major objective of the questionnaire was to address the question: What about the Mopan Mayan Indian lifestyle increases their risk of malaria? Access to this community was gained by discussing this study objective, first with government officials and then with community leaders.

After all residential-like structures were mapped out, a rapid randomization technique was used to select prospective participants. This technique, developed by the World Health Organization (H. Kohler, School of Nursing, U. of MD, Baltimore, personal communication), is designed for household selection in

areas lacking identifiers such as house numbers, streets and zip codes. A bottle was spun at the edge of each neighborhood to randomize the initial household. That household and every third structure thereafter were identified on the map with an 'X' and subsequently invited to participate. Terms of confidentiality were explained and informed consent was gained from the head of household or his spouse (Appendix B). Six households were randomly selected in this fashion and were pilot tested. Final revisions of the tool were then made.

If no one was home at the selected house, the next household was approached. Some families maintained two to three structures, separating cooking and storage from sleeping quarters. In this case the sleeping quarters were viewed to answer the questions pertaining to home construction. Another reason for skipping a house was if the offer to participate was declined. This happened once. However when counting ahead to the next household to be selected, the initial randomization pattern was always used.

If either parent was present and permission was obtained, the interview was conducted. Since the best historical data appeared to be gathered when both husband and wife were available, the interviewers visited the village in the afternoon when the couple was likely to be home. Selected households where only the head female was present were not skipped since this may have biased

the data toward families with men who spent less time working away from home.

The questionnaire was comprised of five sections which were addressed in the following order: housing, mobility, mosquito biting, personal protective measures, and methods of diagnosis and treatment for malaria. Questions ~~were all~~ of the close-ended type except for one: "Please explain any other ways in which your family tries to avoid getting malaria." Skip patterns were incorporated to expedite the interview which lasted from 40 to 80 minutes. Interview length depended primarily on family size since work schedules, patterns of mobility and history of malaria were asked regarding each adult and child.

Interviewers consisted of the principal investigator (PI) and two medical technologists (MT). Interviewing technique was of the semi-standardized type, a mediation between the classic formal and informal approaches (Bennett and Ritchie, 1975). While following a structured order of questions, this allowed the interviewer to reword items and introduce probes to obtain sufficiently detailed answers. For example, "I don't know" was found to be a frequently used phrase whether the interview was in progress or not. As this response was not acceptable, the question was rephrased and/or probes were offered such as: "Had your forth child been borne yet when Feliciana last had malaria?"

Initially, both MTs observed the PI's interviewing style and then were observed by the PI. This was followed by discussion geared towards standardizing the interview process. Completed questionnaires were reviewed by the PI and any ambiguities were addressed with the appropriate interviewer.

Interviews were conducted in English, the second language for the Mopan Mayans. They frequently used their native Kekchi tongue to discuss the details of the questions, but were always able to answer in English. The exception to this rule was found in one neighborhood of Red Bank inhabited by Honduran and Guatemalan immigrants. As they spoke only Spanish, the PI and one MT interviewed them in their language. Regardless of language used, colloquial terms and syntax were substituted whenever possible.

The last area of assessment pertained to each family member's history of malaria. Malaria was defined as coinciding and often repeating symptoms of chills, fever spikes, night sweats, and headache. The respondent often added body aches, nausea, and vomiting to this list but these symptoms were not required for a definition of malaria.

If a family member had not experienced his most recent episode of malaria within the previous twelve months, no further questions were asked regarding that individual. If the opposite were true, then a series of inquiries were made regarding that "recent case"

of malaria. These included date of onset, method of diagnosis, locale of diagnosis, and the specific treatment regime followed, if any.

Statistics of risk and of associations will be calculated once data are entered from questionnaire to computer. Thus far, chi square testing of potential differences in malaria rates between age groups have been calculated. Additionally, a relative risk for malaria between working-aged males and all others has been derived.

III. RESULTS

Thirty-eight families were interviewed. This represents the experience of 197 individuals, 51% female and 49% male. The bulk of participants were between the ages of zero and nine (78), with the next largest group aging from 20 to 44 years (57). The remainder of the sample was distributed as follows: 10 to 19 years (43), 45 to 64 years (14), and age 65 or older (5).

Most people had a history of malaria, with 105 reporting that their last malaria-like episode had occurred within the year. This results in a 53% cumulative incidence (CI). The gender distribution of these recent cases (54% female and 46% male) differed only slightly from the sample.

The age distribution of those reporting malaria within the last year also closely mimicked the overall sample distribution of ages (fig. 4). Slight deviations as compared with the expected CI of 53% were found in the 10 to 19 year group (37%) and in the 20 to 44 year group (61%). However, chi square testing proved these differences to not be statistically significant.

AGE DISTRIBUTION OF CASES AND NON-CASES

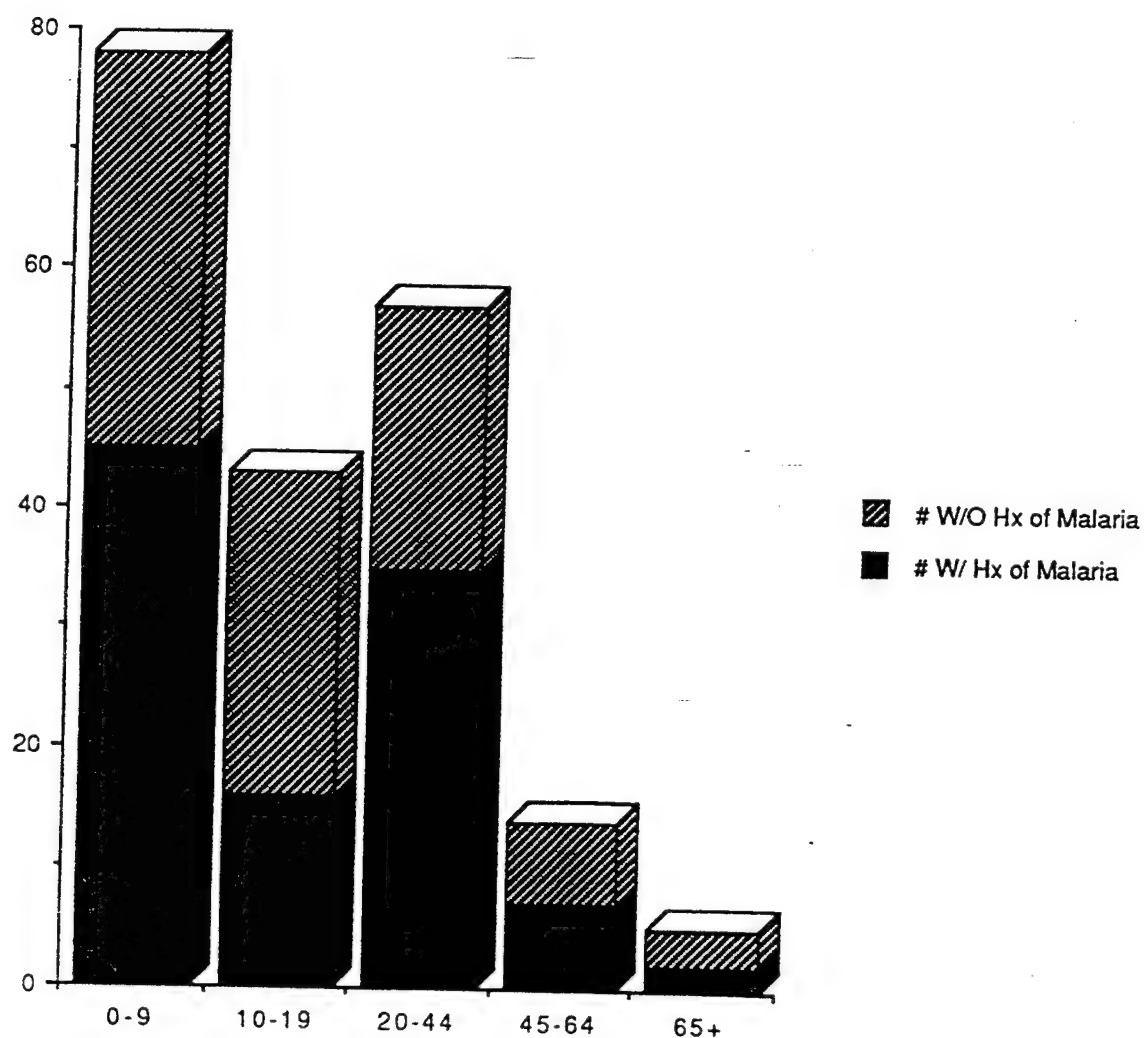


fig. 4

Age

Findings regarding participants' behavior include the following: Families had resided in Red Bank for an average of 6.3 years. The average size of a family was five people. Thirty-seven of the 38 homes, whether thatch or other construction (cement or wood), were not solid and none had screened windows or doors. All participants reported mosquitoes biting indoors between the hours of 6 pm and 5 am. Seventy-two percent of respondents reported that this indoor biting was worse in the wet season with most of the remainder stating that indoor biting was just as bad during the dry season.

With regard to patterns of mobility, the following data were gathered. Ten of 197 (5%) reported having traveled outside the country in the last year. Although specific numbers have not been calculated, men tended to work away from the village from dawn until early or mid-afternoon. Women tended to stay in the village with a few exceptions working occasionally at the banana packaging shed. When asked where each family spent more of the time -indoors or outdoors- between the hours of 6 and 9 pm, most chose indoors for the entire family.

No women and only three men spent evening or nighttime hours away from the village. All respondents reported sleeping indoors and 95% reported bathing in the nearby river or creeks. Baths were invariably taken during hours of low vector activity. Women generally bathed with children during morning hours whereas most

men bathed upon returning from work.

Use of personal protection measures have not been fully tallied but appeared to vary greatly from family to family. Only 2% reported using insect repellent on a routine basis, with 8% stating they applied it occasionally. Bed nets could be observed in most houses but their usage differed not only between families but amongst family members as well. Often it was reported that women and children used netting while the adult men slept in unprotected hammocks, and that far fewer people used netting during the hottest nights.

The final area of results pertain to how those reporting malaria within the past year had their disease confirmed, and what medical treatment they took. Eighty percent of such cases reported that blood smears (via finger stick) were taken from them or their child during the most recent episode of chills, fever spikes, and night sweats. However, it was not uncommon that the case, or their parent, did not recall receiving word of the exam results.

Reporting of medications taken during this recent episode generally fell into two distinct regimes. Forty-three percent reported that they received and took five or more days of anti-malarial medicines. Whereas, 46% said that they received and took one day of medicine on the day of the blood smear. This

latter experience indicates that 'presumptive' treatment was given by the examiner, while the former therapy is considered a more 'radical' treatment. Of the remaining ten cases, eight reported having received no medicines, and two recalled taking three days of medicines (fig. 5).

TREATMENT VARIATIONS AMONG THOSE REPORTING
MALARIAL EPISODE WITHIN PAST 12 MONTHS

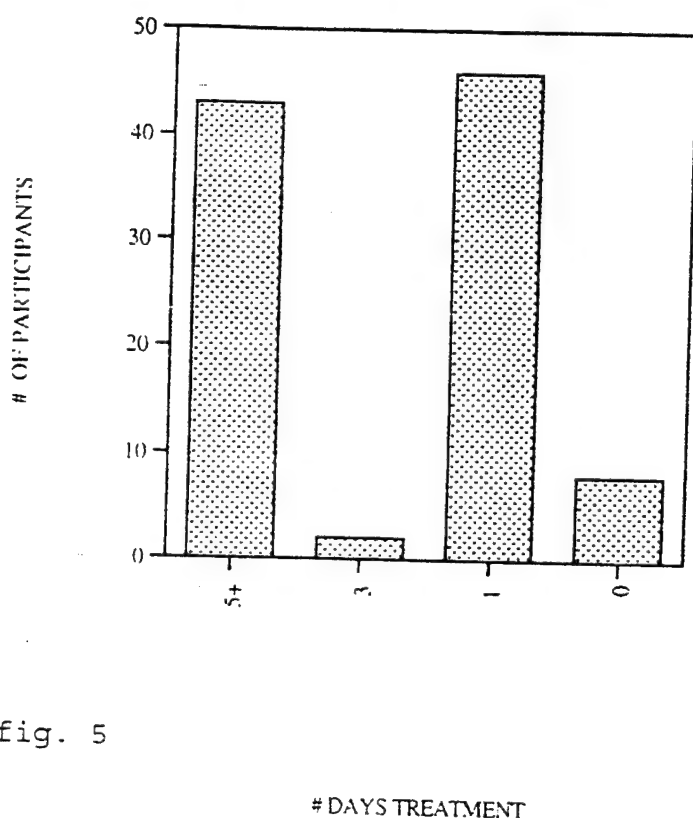


fig. 5

Chloroquine, known to Red Bank residents as "the white pills," is the medicine most commonly used by the villagers. Primaquine ("the brown pills") is slightly less available but is often used in tandem with chloroquine since it kills the liver stages of the plasmodia. Information regarding the number and color of pills

ingested during each day of treatment appeared to be well within the recent memory of most parents. However, this data has not yet been fully analyzed.

Analysis was done to test if the proportions of males and females in the cases were the same as in the sample, for each age group. Chi square tests using observed and expected yielded a non-significant X^2 of 1.59 for females ($p = 0.975$) and a non-significant X^2 of 7.25 for males ($p = 0.4$). The next step in this part of the analysis will be to conduct a power test for proportions to determine the magnitude of differences in age and sex distribution which the study was able to detect. Finally, an overall risk ratio was calculated to test the primary hypothesis of transmission occurring in the village. In a 2×2 table, the incidence of malaria over the past year in working aged men was compared to malaria incidence in all other study participants. This resulted in a risk ratio of 1.20 for men of working age. Using the Mantel-Haenszel Chi formula for count data of sufficient sample size, the confidence limits derived are 0.9 and 1.7. As these include 1.0, the risk ratio of working men to all others appears not only weak, but statistically insignificant.

IV. DISCUSSION

This research was a retrospective cohort study, using randomization to select approximately two-thirds of a highly

malarious community. So far, both strengths and weaknesses in design and implementation have been found. The main hypothesis, that malaria transmission is occurring primarily in the Red Bank village itself, has been confirmed by the study findings. This new information should help focus programmatic efforts toward family-centered control interventions.

The secondary hypothesis, that recrudescence accounts for a considerable portion of malarial episodes in Red Bank, has not been tested. Data may not be of sufficient detail for testing to occur. Had pilot testing been conducted in advance of the actual data collection period, subsequent questionnaire revisions may have better reflected this additional study objective.

It is important to note that *P. vivax* is the predominant vector species in Belize. Since *P. vivax* has a propensity to recur in its human host, independent of new infection, malarial episodes cannot be exclusively defined as incidences of new transmission. Cases derived from this study are, therefore, defined as episodes of classic, malaria-like symptoms. This fact, combined with the inconsistent use and availability of primaquine, should alert the reader to the possibility of considerable rates of recrudescence in Red Bank. Never the less, the resulting 53% cumulative incidence rate approximates the 1993 figure of 43%, reported by the Belizean Government (Vector Control Program, 1994).

Preliminary findings have already been shared with the community of Red Bank, the Belize Vector Control Program, and local providers of health care. Recommendations stemming from the analysis of this study will be disseminated as well and will likely include the following points. Re-starting Anopheles population control by indoor insecticide spraying would have a great impact in Red Bank. Due to the open construction of houses, coupled with the finding that almost all inhabitants are at home during the peak biting hours, resumption of spraying would seem an important first step toward malaria control.

Interested villagers, called "volunteer coordinators" (VCs), are identified by the staff of the Vector Control Program for the Stann Creek District. These VCs are often, but not always, given formal training in diagnostic and therapeutic malarial protocols. As well, they are provided with chloroquine and primaquine and any blood slides they prepare are collected and examined in Dangriga, the program headquarters. Increased training of village VCs and access to adequate supplies of chloroquine and primaquine are needed in Red Bank. One VC (self-reported as untrained) was found to be dispensing three chloroquine every four hours for one day of treatment. Other deviations from recommended dosages, such as the omission of primaquine, can result in equally ineffective treatment. In those who have had malaria before, and who could therefore be experiencing recrudescence, a 14 day treatment of primaquine is currently

recommended. This regime was rarely found to be the case in Red Bank, although most had malaria several times over.

The villagers of Red Bank were often found to exhibit a high degree of concern and animation when discussing malaria, quite distinct from their normally passive manner. This grass roots interest should be capitalized upon by targeting Red Bank with Public Health Education regarding prevention, diagnosis, and treatment of malaria. Although labor-intensive at first, these efforts might soon lead to villagers educating each other and their children regarding hours of high risk, proper pill-taking, cardinal signs of infection, and more. The Vector Control Team attempts to visit Red Bank weekly, roads, vehicles, and time permitting. Community Health Nurses also visit the community every six weeks, primarily for the purpose of well child and pre-natal care. Other than these two programs, there is no available health care, which makes health education an appropriate option. The level of empowerment gained through public health education often leads to a smaller financial investment by the local and federal governance.

Whether or not implementation of the above three recommendations occurs, the malaria problem in Belize will continue to be documented and studied. In further studying the occurrence of malarial episodes, it would seem helpful to compare migrant and Mayan communities to communities less affected. The goal of

these efforts should not be to incriminate non-natives or Indians as "carriers of malaria", as malaria is far too common for importation to be of valid concern. Rather, the goal of further behavioral studies could be to determine what is different (and conversely, the same) between communities radically differing in malarial rates. Are type of housing, level of acculturation, socio-economic status, or degree of access to health care accurate indicators of a community's ability to keep their malaria under control? Answers to these questions could appropriately direct programmatic involvement.

In addition to further behavioral host studies, more information regarding the vector species is needed. Many of their breeding and feeding habits are already known, but continued surveillance for chloroquine-resistant strains of plasmodia will be needed. In addition, when spraying is resumed, its effectiveness in either killing or repelling the various species of mosquitoes will need to be determined.

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I. ENVIRONMENT / LIVING CONDITIONS

PART A: INTERVIEWER OBSERVATIONS

1. HOUSE ID _____ DATE ____/____/____
2. What are walls made of?
_____ planks or trees with spaces _____ wood sheets w/o spaces
_____ aluminum, tin _____ pimento trees _____ clay
_____ other, please describe _____
3. What is roof made of?
_____ wood _____ aluminum, tin _____ adobe/clay
_____ thatch _____ other, please describe _____
4. What is floor made of? _____ wood _____ concrete _____ dirt
5. Is there a door? _____ yes _____ no
6. Is door solid? _____ yes _____ no
7. Is the door screened? _____ yes _____ no
8. Are there windows? _____ yes _____ no
9. Are the windows screened? _____ yes _____ no _____ some
10. Do windows have shutters? _____ yes _____ no
11. Are shutters solid? _____ yes _____ no
12. Can shutters be closed? _____ yes _____ no
13. How far is this house from the nearest permanent stream?
(Permanent means it contains water all year.)
_____ # of meters

PART B. QUESTIONS TO A HEAD OF HOUSEHOLD

1. How many people are in this household? _____

[illegible]

HOUSE ID _____

1. How long have you lived in Red Bank? _____ yrs
2. During what year did your family move into this house?
_____/_____/_____
3. Where does the family usually bathe?
_____ river _____ indoors _____ outside the house
_____ village pump
4. At home, does anyone sleep outdoors and how many nights per week?
who: _____ # nights/week
_____ # nights/week
_____ # nights/week
5. At home, who uses a bed net (pavajon)?
_____ always occasional treated
everyone except _____
6. Do any of you travel outside the village? _____ yes _____ no
who: _____
where: _____
how often: _____ #/mo. time of day gone: _____
7. Who has travelled outside of Belize since July 1993? _____ no one
who: _____
where: _____
8. Are you bitten by mosquitoes indoors? _____ yes _____ no
9. If so, when do you have the greatest problem with indoor biting?
_____ all year _____ wet season _____ dry season
10. What time of day do you usually get bitten indoors?
_____ morning (5 am - 12 noon) _____ afternoon (12 noon - 6 pm)
_____ evening (6 pm - 8 pm) _____ nighttime (8 pm - 5 am)
11. Is your sleep frequently disturbed by mosquitoes?
_____ yes _____ no
12. If you work at a work site, where do you get mosquito-bitten more?
_____ at home _____ at work site
13. Does anyone here use a spray or cream on their body to keep mosquitoes off?
_____ usually _____ occasionally _____ never; when _____
14. Please explain any other ways in which your family tries to avoid getting malaria.

II. EMPLOYMENT OUTSIDE HOME House ID _____

Name _____ (HOH)

MILPA

PKG. SHED

BANANA FIELD

OTHER

Work? _____

Start Date _____

Sleep Over? _____

mon

(# nights/month)

work after 5pm _____

or before 5am? _____

mon

mon

mon

Name _____

MILPA

PKG. SHED

BANANA FIELD

OTHER

Work? _____

Start Date _____

Sleep Over? _____

mon

(# nights/month)

work after 5pm _____

or before 5am? _____

mon

mon

mon

Name _____

MILPA

PKG. SHED

BANANA FIELD

OTHER

Work? _____

Start Date _____

Sleep Over? _____

mon

(# nights/month)

work after 5pm _____

or before 5am? _____

mon

mon

mon

IF NOT SLEEPING AWAY AT WORK, SKIP TO NEXT SECTION

When you sleep away from home at your work,

3. . . . is there a river nearby? _____ yes _____ no

4. . . . where do you bathe? _____ don't bathe

_____ river _____ indoors _____ other (describe) _____

5. . . . are you sleeping indoors?

_____ yes _____ no If indoors, skip to next section.

6. . . . is it just like this house? _____ yes _____ no

7. . . . is your secondary dwelling screened? _____ yes _____ no

8. . . . do you use a bed net?

_____ always _____ occasionally _____ never

9. . . . if you use a bed net, has it been treated with insecticide?

_____ yes _____ no _____ do not know

House 1D

1. Have you ever had malaria? yes no
2. If so, how many times have you had malaria?
3. When did you last have malaria? /
4. Did you have... fever? chills? sweating at night?
 head ache? ...any other symptoms?

5. Do you presently have malaria? _____ yes _____ no

6. Who confirmed your malaria? _____ V.C.: _____ (name)
_____ doctor/nurse
_____ malaria man from Dangriga

by other, explain _____

7. Where were you when the diagnosis was made?
 _____ in village _____ outside village. where? _____

8. Did they stick your finger for blood? _____ yes _____ no

9. Did they give you any medicines? _____ yes _____ no

10. If so, how many white ones? _____
 how many brown ones? _____

11. Did you take all the pills that day? _____ yes _____ no

12. Did you get the results from your blood test? _____ yes _____ no

13. If so, were the results positive for malaria? _____ yes _____ no

14. Did you they then give you more pills to treat your malaria?

DAY	1	2	3	4	5	6
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PRIMA.

15. Was the treatment completed? ☐ yes ☐ no
16. Have you ever had yellow jaundice? ☐ yes ☐ no
17. If so, when did you have it? / /

1. Have you ever had malaria? yes no
2. If so, how many times have you had malaria?
3. When did you last have malaria? /
4. Did you have... fever? chills? sweating at night?
 head ache? ...any other symptoms?

5. Do you presently have malaria? _____ yes _____ no

6. Who confirmed your malaria? _____ V.C.: _____ (name)
_____ doctor/nurse
_____ malaria man from Dangriga
by other, explain _____

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10. If so, how many white ones? _____
 how many brown ones? _____
11. Did you take all the pills that day? _____ yes _____ no
12. Did you get the results from your blood test? _____ yes _____ no
13. If so, were the results positive for malaria? _____ yes _____ no
14. Did you they then give you more pills to treat your malaria?

DAY 1 2 3 4 5 6

CHLOR.

PRIMA.

15. Was the treatment completed? _____ yes _____ no
16. Have you ever had yellow jaundice? _____ yes _____ no
17. If so, when did you have it? _____/_____/_____

XX

NAME _____

1. Have you ever had malaria? _____ yes _____ no
2. If so, how many times have you had malaria? _____
3. When did you last have malaria? _____/_____/_____
4. Did you have... fever? _____ chills? _____ sweating at night?
 _____ head ache? ...any other symptoms? _____

NOTE: IF THIS LAST CASE OF MALARIA IS WITHIN THE LAST YEAR-CONTINUE;
 OTHERWISE---- Skip to #15

5. Do you presently have malaria? _____ yes _____ no
6. Who confirmed your malaria? _____ V.C.: _____ (name)
 _____ doctor/nurse
 _____ malaria man from Dangriga
 by other, explain _____
7. Where were you when the diagnosis was made?
 _____ in village _____ outside village, where?
8. Did they stick your finger for blood? _____ yes _____ no
9. Did they give you any medicines? _____ yes _____ no
10. If so, how many white ones? _____
 how many brown ones? _____
11. Did you take all the pills that day? _____ yes _____ no
12. Did you get the results from your blood test? _____ yes _____ no
13. If so, were the results positive for malaria? _____ yes _____ no
14. Did you they then give you more pills to treat your malaria?

CHLOR.

PRIMA.

15. Was the treatment completed? yes no
16. Have you ever had yellow jaundice? yes no
17. If so, when did you have it? / / /

NAME _____

1. Have you ever had malaria? _____ yes _____ no
2. If so, how many times have you had malaria? _____
3. When did you last have malaria? _____
4. Did you have... _____ fever? _____ chills? _____ sweating at night?
_____ head ache? ...any other symptoms? _____

NOTE: IF THIS LAST CASE OF MALARIA IS WITHIN THE LAST YEAR-CONTINUE;
OTHERWISE---- Skip to #15

5. Do you presently have malaria? ☐ yes ☐ no
6. Who confirmed your malaria? ☐ V.C.: _____ (name)
☐ doctor/nurse
☐ malaria man from Dangriga
by other, explain : _____
7. Where were you when the diagnosis was made?
☐ in village ☐ outside village, where? _____
8. Did they stick your finger for blood? ☐ yes ☐ no
9. Did they give you any medicines? ☐ yes ☐ no
10. If so, how many white ones? _____
how many brown ones? _____
11. Did you take all the pills that day? ☐ yes ☐ no
12. Did you get the results from your blood test? ☐ yes ☐ no
13. If so, were the results positive for malaria? ☐ yes ☐ no
14. Did you they then give you more pills to treat your malaria?

DAY	1	2	3	4	5	6
-----	---	---	---	---	---	---

CHLOR.

PRIMA.

15. Was the treatment completed? _____ yes _____ no
16. Have you ever had yellow jaundice? _____ yes _____ no
17. If so, when did you have it? _____

SEP 27 1993

Donald R. Roberts
301-295-3734

VARIATION IN THE HINDTARSAL MARKINGS OF *ANOPHELES DARLINGI* (DIPTERA: CULICIDAE) IN BELIZE

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ABSTRACT. Aberrant phenotypes of *Anopheles darlingi* with basal dark scaling on either one or both of hindtarsomeres 3 and 4 are reported from Belize. Based on wild-caught females and adults reared from wild-caught larvae, it appears that approximately 8% of the natural population bears some degree of basal dark scaling on these hindtarsomeres. The occurrence of similar variants in other species of the subgenus *Nyssorhynchus* is summarized, and their significance in terms of inaccurate species identification is noted.

INTRODUCTION

Anopheles (Nyssorhynchus) darlingi Root has not been reported or collected from Belize since Komp (1940, 1941), Kumm and Ram (1941), and Walker (unpublished³). In fact, we thought this species had disappeared from Belize, perhaps due to agricultural and control practices, because we did not encounter it during intensive larval surveys conducted throughout the country in September 1990, April 1991, and September 1992 (Rejmankova et al. 1993, Roberts et al. 1993). In May 1993, however, we repeatedly collected adult females of this species from human bait both inside and outside houses located near rivers between Middlesex in Stann Creek District and Belmopan in Cayo District. Larvae were subsequently collected from riverine habitats near St. Thomas and Belmopan. *Anopheles darlingi* undoubtedly contributes in a major way to the increasing

numbers of malaria cases in Belize (PAHO 1992).

Faran and Linthicum (1981) and Linthicum (1988) used the absence of dark scaling on hindtarsomeres 3 and 4 as the primary character for distinguishing adults of the subgenus *Nyssorhynchus* from those belonging to the other subgenera of *Anopheles* in the Neotropical Region. They indicated that hindtarsomeres 3 and 4 are entirely white in this group, except in "unusual variants," which apparently refers to the so-called "mutants" and "anomalous specimens" of *An. albimanus* Wiedemann, *An. aquasalis* Curry, *An. strodei* (Root), and *An. triannulatus* (Neiva and Pinto) discussed by Faran (1980). Linthicum (1988) specifically listed *An. rondoni* (Neiva and Pinto) and *An. nigratarsis* (Chagas) as exceptions, but these species differ from the others in having basal dark bands constantly present on hindtarsomere 3 (*An. rondoni*) or both hindtarsomeres 3 and 4 (*An. nigratarsis*).

Faran (1980) divided the subgenus *Nyssorhynchus* into two sections, the *Albimanus* and *Argyritarsis* sections, but excluded four poorly known species of the "*Myzorhynchella* group," which was raised only recently to sectional status (Peyton et al. 1992). He distinguished adults of the *Argyritarsis* Section from those of the *Albimanus* Section primarily by the absence of a dark basal band on hindtarsomere 5. *Anopheles darlingi* is a member of the *Argyritarsis* Section, which is characterized by having hindtarsomeres 3-5

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³ Four females of *An. darlingi* from Sierra de Agua (17° 30' N 88° 50' W), Orange Walk District, collected by A.J. Walker in June 1946 are deposited in the National Museum of Natural History, Smithsonian Institution. These specimens were examined and listed by Linthicum (1988).

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entirely white-scaled (Faran 1980, Linthicum 1988). Of the eight species belonging to the *Argyritarsis* Section, dark markings on hindtarsomeres 3 and 4 have been observed only in *An. darlingi*. Most of the species mentioned above belong to the *Albimanus* Section. *Anopheles nigratarsis* belongs to the *Myzorrhynchella* Section.

Komp (1942:37) mentioned that he had "specimens of *darlingi* from British Honduras [= Belize] with additional black hindtarsal bands," but this observation apparently went unnoticed by later authors because Komp never included it in a species description. We first noticed the presence of basal dark scaling on hindtarsomeres 3 and 4 in a few specimens of *An. darlingi* while identifying mosquitoes collected during malaria vector ecology studies involving the use of remote sensing. These mosquitoes were all frozen for *Plasmodium* detection and identification. The purpose of this paper is to bring attention to the presence of basal dark markings observed in adults of *An. darlingi* reared from wild-caught larvae and the progeny of wild-caught females that were frozen for isozyme analysis. There are no published reports of aberrant hindtarsal markings in populations of *An. darlingi* from any other country in Central or South America.

MATERIALS AND METHODS

Hindtarsal markings were examined in adults reared from wild-caught larvae and progeny broods obtained from females captured on human bait on May 25, 1993. Larvae were collected in shaded masses of floating plant debris and patches of *Cabomba* sp. along the edges of the Sibun River near St. Thomas (17° 09' N 88° 37' W) and Roaring Creek near Belmopan (17° 15' N 88° 48' W). The specimens were transported the next day to the Smithsonian Institution Museum Support Center in Suitland, MD. Larval collections and progeny broods were reared separately in plastic pans in an air-conditioned room held at $21 \pm 1^\circ\text{C}$. Each pan was provided with straw for floatage and gently aerated (through a sandstone) by means of an

aquarium pump. Fourth-instar larvae and pupae were removed from the pans and reared individually in plastic vials. Most of the adults reared from wild-caught larvae and a portion of those reared from each progeny brood were mounted on points on pins and examined for hindtarsal markings. All of these experiments, along with their associated larvae and/or pupal exuviae, were deposited in the National Museum of Natural History, Smithsonian Institution, Washington, DC, for future study and reference. A number of adults (12%) obtained from progeny broods died upon emergence and could not be saved as voucher specimens. These specimens were examined for hindtarsal markings, then discarded along with their associated larval and pupal exuviae. The remainder of the adults (24% from wild-caught larvae and 39% from progeny broods) were examined while alive and subsequently frozen at -70°C for later biochemical studies. We retained larval and pupal exuviae from the frozen adults reared from wild-caught larvae. Two or three first-, second-, third-, and fourth-instar larvae from each progeny brood were preserved in 80% ethanol for future morphological study.

OBSERVATIONS

The variation observed in the hindtarsal markings of *An. darlingi* from Belize is illustrated in Fig. 1. Figure 1A shows the normal condition where the distal portion of hindtarsomere 2 and all of hindtarsomeres 3–5 are white-scaled. The other drawings in Fig. 1 illustrate variation in basal dark scaling sometimes present on the third and fourth hindtarsomeres: (1) dark scaling at the base of hindtarsomere 3 (Fig. 1B), (2) dark scaling at the base of hindtarsomere 4 (Fig. 1C), and (3) dark scaling at the bases of both hindtarsomeres 3 and 4 (Fig. 1D,E). The amount of dark scaling on hindtarsomeres 3 and 4 is variable, ranging from a narrow or incomplete ring to a broad, distinct band. The dark scaling, when present, usually forms complete bands. In general, when dark scaling is well developed on hindtarsomere 3, there is a corresponding reduction in the amount of

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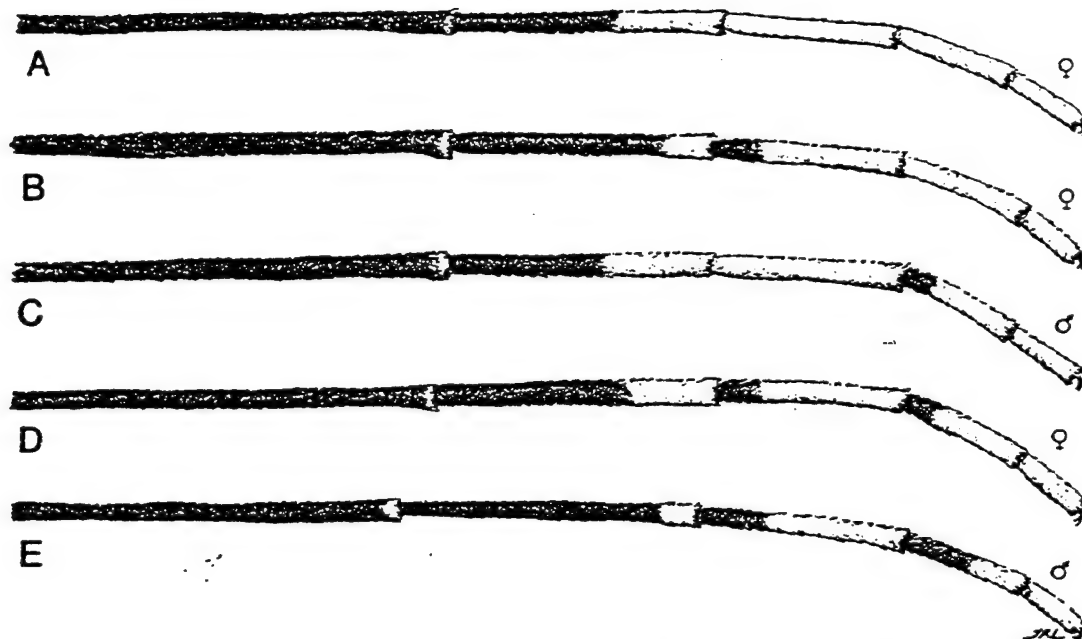


Fig. 1. Hindtarsal markings observed in specimens of *Anopheles darlingi* from Belize. A, Prevalent form, hindtarsomeres 3-5 entirely white-scaled; B-E, aberrant forms showing variation in basal dark scaling that sometimes occurs on one or both of hindtarsomeres 3 and 4.

white scaling on the distal portion of hindtarsomere 2 (Fig. 1B,E). In such cases, the extent of basal dark scaling on hindtarsomere 2 exceeds the range of "basal 0.35-0.55 dark" given by Linthicum (1988).

Ninety-four adults were reared from wild-caught larvae. Seven of these had basal dark scaling on one or both of hindtarsomeres 3 and 4. Six specimens (4♂, 2♀) exhibited the condition shown in Fig. 1C, i.e., basal dark scaling on hindtarsomere 4, and one female had basal dark scaling on both hindtarsomeres 3 and 4. If this sample is representative of the natural population of *An. darlingi* in Belize, then 7.4% (7/94) of the wild population would be expected to have dark markings on one or both hindtarsomeres 3 and 4. Of the seven individuals, 85.7% (6/7) had dark scaling at the base of hindtarsomere 4, and 14.3% (1/7) had dark scaling on both hindtarsomeres 3 and 4. Of the overall sample, 6.4% (6/94) had dark scaling at the base of hindtarsomere 4, and 1.1% (1/94) had dark scaling on the bases of both hindtarsomeres 3 and 4.

Progeny broods were obtained from 11 females. One female had a prominent dark band at the base of hindtarsomere 3, another had prominent dark basal bands on both hindtarsomeres 3 and 4, and the remainder (9) had normal hindtarsi. If these wild-caught females are grouped with the 94 adults reared from wild-caught larvae, then a total of 8.6% (9/105) of the natural population of *An. darlingi* in Belize would be expected to have some degree of dark scaling on hindtarsomeres 3 and 4. Two females among these individuals (1.9% of 105) had dark scaling on the bases of both hindtarsomeres 3 and 4, one female (1.0%) had dark scaling only at the base of hindtarsomere 3, and six individuals (4♂, 2♀) (5.7%) had dark scaling only on the base of hindtarsomere 4.

Of the progeny obtained from the female with dark scaling at the base of hindtarsomere 3, only nine survived to adulthood. One female was exactly like the mother in having dark scaling at the base of hindtarsomere 3, and one male differed in having dark scaling at the bases of both hindtarsomeres 3 and 4.

Three males and four females exhibited the normal condition shown in Fig. 1A. A total of 22.2% (2/9) of the individuals that survived from this brood had dark bands on the hindtarsomeres.

Only three offspring (2♂, 1♀) of the female with prominent dark bands at the bases of hindtarsomeres 3 and 4 survived to adulthood. Unfortunately, the single female emerged from the pupal exuviae without hindtarsi. These were left within the exuviae and could not be examined. The two males, however, had basal dark bands on hindtarsomeres 3 and 4 exactly like their mother. It is possible that this brood may have bred true, i.e., all of the progeny may have exhibited the maternal phenotype.

Of the progeny obtained from the other nine females, 119 (57♂, 62♀) survived to adulthood, and all but one of these were normal with respect to hindtarsomeres 3 and 4. A single female from a brood of 12 surviving adults (6♂, 6♀) had dark markings on the bases of hindtarsomeres 3 and 4. Therefore, 8.3% (1/12) of this brood exhibited dark hindtarsal markings. This is nearly the same ratio seen in wild-caught specimens.

DISCUSSION

Accurate identification of mosquitoes depends on a thorough knowledge of morphological variation within species. Variation in ornamentation sometimes leads to erroneous identification. In fact, lack of knowledge of variation in hindtarsal markings has led to the naming of a number of varieties and species that are conspecific, e.g., the *bisignatus* and *trisignatus* varieties of *An. albimanus* (Hoffmann 1938), the *guarauno* and *delta* varieties of *An. aquasalis* (Anduze 1948), *An. deltaorinoquensis* Cova Garcia, Pulido F. and Amanista M., which is a synonym of *An. aquasali* (Faran 1980), and *Cellia cuyabensis* (Neiva and Pinto), which is a synonym of *An. triannulatus* (Pinto 1939). Because morphological keys are designed by trained taxonomists primarily for the use of non-taxonomists and inexperienced identifiers, it is important that keys account for intraspecific

variation as well as interspecific differences. In most published keys, specimens of *An. darlingi* with dark markings on hindtarsomeres 3 and 4 will key properly to the correct subgenus and species, but this is not so with the keys of Faran and Linthicum (1981) and Linthicum (1988), where reliance on the first or primary key character would cause these specimens to be misidentified at the subgeneric level. Fortunately, *An. darlingi* is a very distinct species in Belize, where it usually should be recognizable in both the adult and larval stages.

Among the subgenera of *Anopheles* in the Neotropical Region, hindtarsomeres 3 and 4 are not entirely white in *Anopheles*, *Lophopodomyia*, *Kerteszia*, and *Stethomyia*. As indicated above, these hindtarsomeres are entirely white in species of the *Nyssorhynchus* except for *An. nigratarsis* (Myzorhynchella Section), *An. rondoni* (Albimanus Section), and "unusual variants" (Faran and Linthicum 1981, Linthicum 1988). *Anopheles nigratarsis* is characterized by the constant presence of basal dark bands on hindtarsomeres 3 and 4 and *An. rondoni* by the constant presence of a dark basal band on hindtarsomere 3. Aberrant dark bands sometimes occur variously on one or more of hindtarsomeres 3–5 in certain populations of *An. albimanus*, *An. aquasalis*, *An. strodei*, and *An. triannulatus* of the Albimanus Section and *An. darlingi* of the Argyritarsis Section. A summary of additional dark bands observed in these species is given in Table 1. These are the variants that are likely to cause problems for non-taxonomists, particularly those who place too much emphasis on primary key characters, use them out of habit, or use them because they are more discrete and easier to observe than secondary key characters.

The phenotypes with aberrant hindtarsal bands observed in species of *Nyssorhynchus* have been called "mutants" and "anomalous specimens" by various authors (e.g., Rozeboom 1963, Kitzmiller and Mason 1967, Faran 1980). Indications are that these phenotypes are fairly common, especially in *An. albimanus*, *An. triannulatus*, and *An. darlingi*. From this study, it appears that approx-

Table 1. Summary of aberrant hindtarsal markings observed in species of the subgenus *Nyssorhynchus* of *Anopheles*.

Section	Species	Variants	Populations from	Principal references
Albimanus	<i>albimanus</i>	Basal dark bands on: 1) hindtarsomere 3 2) hindtarsomeres 3,4	Costa Rica, El Salvador, Guatemala, Texas (U.S.A.)	Hoffmann 1938, Rozeboom 1963, Faran 1980
	<i>aquasalis</i>	Apical dark bands on: 1) hindtarsomere 4 2) hindtarsomeres 3,4 Basal dark bands on: 3) hindtarsomeres 3,4	Venezuela	Anduze 1948, Faran 1980
	<i>strodei</i>	Basal dark bands on: 1) hindtarsomeres 3,4 2) hindtarsomere 4 3) hindtarsomeres 3,4 but absent on 5 ¹	Brazil	Rachou and Ferraz 1951
	<i>triannulatus</i>	Basal dark bands on: 1) hindtarsomere 4 2) hindtarsomeres 3,4	Brazil	Pinto 1939, Galvão and Lane 1941
Argyritarsis	<i>darlingi</i>	Basal dark bands on: 1) hindtarsomere 3 2) hindtarsomere 4 3) hindtarsomeres 3,4	Belize	present study

¹ Species of the *Albimanus* Section are characterized by the presence of a basal dark band on hindtarsomere 5.

imately 8% of the *An. darlingi* in Belize have basal dark scaling on either one or both of hindtarsomeres 3 and 4, and the data suggest that a heritable genetic basis exists for the expression of this trait. Consequently, we prefer to characterize these phenotypes as "aberrant forms" or "normal variants," which imply deviation from the usual or prevalent form rather than a rare individual or strain resulting from mutation.

larvae; to Jim Pecor and E.L. Peyton, WRBU, for assistance in setting up collections and rearing specimens; to Ronald A. Ward, Walter Reed Army Institute of Research, Washington, DC, and E.L. Peyton for commenting on the manuscript; and to Jayson Glick, Manager, WRBU, for allowing us the use of laboratory space and equipment needed to rear and process specimens.

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Initial report of a hepatitis investigation in rural Belize

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Abstract

In spring 1991, Belizean health officials expressed concern about a possible hepatitis outbreak in a banana farming district. A study was designed to identify cases and to address the serological prevalence of hepatitis virus markers. Three populations were studied: (i) persons meeting a clinical case definition for hepatitis; (ii) designated banana workers; and (iii) people in a random sample of households in the community. Information was collected using questionnaires and sera were collected for laboratory testing. This report presents the preliminary results of a study conducted in June 1991. Among people who met the clinical case definition, 24% of 42 tested had immunoglobulin M antibody to hepatitis B virus (HBV) core antigen (anti-HBc IgM). In the worker and household survey populations, 284 and 280 people, respectively, were tested for anti-HBc IgM. In each group, 4% were positive. HBV surface antigen was found in 37% of 43 clinical cases, 18% of workers, and 13% of people in the household survey. Among the 3 study populations, the prevalence of HBV core antibody (anti-HBc) ranged from 73% to 81%. Almost all tested persons had evidence of prior hepatitis A virus infection. Evidence of prior infection with hepatitis viruses A and B was widespread, but an aetiology could not be established for most of the clinical cases. However, the prevalence of hepatitis B markers in this population was very high compared to other reports from the Caribbean.

Introduction

In May 1991, concern that cholera might be spreading north through Central America prompted officials of the Belize Ministry of Health to initiate a cholera prevention and control campaign. In the Cowpen area of south-east Belize, there was no report of illness resembling cholera; however, banana farm workers and local health officials expressed concern about allegations that large numbers of people were or had been ill with hepatitis. Some people had diagnosed themselves as having hepatitis, based upon the occurrence of jaundice. In others, the diagnosis had been made by local health professionals.

Cowpen residents considered the appearance of hepatitis to be an annual occurrence associated with the dry season; they attributed the disease to poor water quality. However, many believed that the 1991 dry season had been more severe and prolonged than usual and that the number of hepatitis cases, particularly in young adults, was greater than that observed in past years. Concern heightened when a young pregnant woman allegedly died with hepatitis.

When the Belizean Ministry of Health requested assistance in investigating the alleged outbreak, we conducted a preliminary investigation in May 1991 to establish the cause(s) of the reported illnesses. The May investigation confirmed the presence of hepatitis in the Cowpen area, based upon abnormal liver function tests and serological studies for hepatitis virus markers. This was followed in June 1991 by an expanded effort to identify cases and to make a cross-sectional study for hepatitis markers in banana farm workers and their families. This is the initial report of the June 1991 investigation. Serological studies and data analyses are continuing, and results of work still in progress will be reported later.

Materials and Methods

Three study populations were defined: cases, banana workers, and households. Individual, household and farm questionnaires were developed in English and Spanish for the uniform collection of information. All individuals were given unique identifiers, which were assigned to serum collection tubes and questionnaires.

A case of hepatitis was defined as anyone who, in the preceding 6 months, based upon the review of a study team physician, had been diagnosed as having hepatitis

by a health care worker or had experienced jaundice. Possible cases were identified for review by a team physician through (i) local health worker reports of currently or previously ill people, (ii) reports from farm and civic leaders of ill people, (iii) study team physicians holding sick call and making house calls on request, and (iv) study team members asking every adult contacted if they had been jaundiced or diagnosed as having hepatitis, or knew of anyone who had. After a study team physician determined that a person met the case definition, serum was collected and a case questionnaire was used to obtain illness data not covered in other questionnaires.

During May-June 1991, local officials in Cowpen estimated the total population of banana farm managers, banana field and shed workers, and family members to be 1300 to 1500 people. These people worked on 7 different banana farms and lived in 8 worker housing areas, all of which were identified for study. Farm personnel usually were studied at the farm packing shed and were questioned about demographic variables, the occurrence of illness, and variables associated with hepatitis virus transmission. An attempt was made to study all banana farm workers. However, 2 factors limited the percentage studied: (i) when banana harvesting and packing were occurring at high intensity, many workers could not be released to participate in the study; and (ii) when there was a lull in harvesting and packing, many workers immediately left the area to visit friends and relatives in Belize or in their homeland. At each household, a household census with demographic information was obtained, and questions were asked about the occurrence of illness and variables associated with hepatitis virus transmission.

Blood for serological studies was obtained from all consenting individuals over 4 years of age. Four years was established as the cut-off age because of reluctance on the part of most parents to have blood drawn from younger children. Response refusals were rare.

An attempt was made to test all sera for antibody to hepatitis A virus (anti-HAV), immunoglobulin M (IgM) antibody to hepatitis A virus (anti-HAV IgM), hepatitis B virus (HBV) surface antigen (HBsAg), antibody to HBV core antigen (anti-HBc), IgM antibody to HBV core antigen (anti-HBc IgM), and antibody to hepatitis C virus (anti-HCV). Only sera positive for HBsAg were tested for HBV e antigen (HBeAg) and antibody to hepatitis delta virus (anti-HDV). All sera were

from cases in which there was no serological evidence of infection with any of the hepatitis viruses also were tested by enzyme-linked immunosorbent assay (ELISA) for IgG and IgM antibodies against leptospira and 2 strains of hantavirus.

Anti-HAV, anti-HAV IgM, and anti-HDV were determined using ELISA kits from Abbott Laboratories (North Chicago, Illinois, USA). Testing associated with HBV also was done using Abbott ELISA kits. Ortho Diagnostics (Raritan, New Jersey, USA) ELISA kits were employed to determine the presence of antibody to HCV. Both the first generation anti-HCV tests and the second generation immunoblot assay for the detection of antibody to HCV (RIBA-HCV) were utilized. Antibody to HEV was detected using a prototype ELISA GOLDSMITH *et al.*, 1992; SKIDMORE *et al.*, 1992).

Data were entered into an EpiInfo database (DEAN *et al.*, 1990). Check files and double entry were used to ensure valid transfer of hard copy questionnaires to the data files. EpiInfo was also used to develop descriptive statistics.

Results

A summary of the serological testing results for hepatitis markers is presented in the Table. The clinical case, worker and household survey populations were not mutually exclusive.

77%) had anti-HBc, indicating prior HBV infection.

Of 482 inhabited dwellings in the 8 housing areas, 146 (30%) were randomly selected and studied. The household sample subgroup included 285 people. The mean age was 26 years (SD=15); 152 (53%) were males. Almost all (275, 98%) had anti-HAV. The 2 children with recent HAV infection described in the clinical case group are also shown in the household sample in the Table. Ten (4%) had anti-HBc IgM, and 37 (13%) had HBsAg. Twenty (54%) people with HBsAg also had HBeAg. As in the other subgroups, most (203, 73%) had evidence of past HBV infection.

In all population subgroups studied, only very small numbers of people were found to have had contact with HCV or HEV. There was no evidence of exposure to HDV or recent infection with leptospira or hantavirus.

Discussion

We found a high prevalence of people with evidence of prior infection with HAV and HBV, a high prevalence of HBsAg positivity, and evidence for recent HBV infection in 24% of the people with clinical illness. Overall, evidence of HBV infection was found in 73% of the people studied, and 16% were positive for HBsAg. The prevalence of anti-HCV was low, and no evidence was found for HDV infection.

Nearly all people studied had anti-HAV. In contrast,

Table. Summary of results of serological testing for hepatitis markers, Cowpen, Belize, 1991

Specific marker ^a	Clinical cases n=47		Banana workers n=290		Household sample n=285	
	No. tested	No. positive	No. tested	No. positive	No. tested	No. positive
Anti-HAV	43	41 (95%)	284	277 (97%)	280	275 (98%)
Anti-HAV IgM	43	2 (5%)	282	0	278	2 (1%)
HBsAg	43	16 (37%)	284	50 (18%)	280	37 (13%)
HBeAg ^b	15	4 (27%)	49	13 (26%)	37	20 (54%)
Anti-HBc	42	34 (81%)	284	219 (77%)	278	203 (73%)
Anti-HBc IgM	42	10 (24%)	284	12 (4%)	280	10 (4%)
Anti-HCV	43	1 (2%)	284	3 (1%)	280	3 (1%)
Anti-HDV ^b	14	0	48	0	32	0
Anti-HEV ^c	38	2 (5%)	24	2 (8%)	18	0

^aHAV=hepatitis A virus, IgM=immunoglobulin M, HBsAg=hepatitis B virus surface antigen, HBeAg=hepatitis B virus e antigen, HBc=hepatitis B virus core antigen, HCV=hepatitis C virus, HDV=hepatitis D virus, HEV=hepatitis E virus. Insufficient quantities of sera prevented complete testing of all people studied.

^bOnly people positive for HBsAg were tested. Expressed percentages are based upon the number of HBsAg positive people tested.

^cOnly clinical cases were tested. Some cases were workers; some were also part of the random household sample.

Forty-seven people met the clinical case definition of hepatitis. Their mean age was 26 years (SD=14); 29 (62%) were males. Twenty-nine cases were identified as workers, and 25 (86%) were male. The average age of worker cases was 32 years (SD=11).

Laboratory results were obtained for 43 cases. Two children were too young for drawing blood and 2 serum specimens were lost. Forty-one (95%) of the cases were positive for anti-HAV, and 2, both children, had anti-HAV IgM, indicating recent HAV infection. Ten (24%) were newly infected with HBV, as demonstrated by the presence of anti-HBc IgM, and 16 (37%) were HBsAg positive. Among those with HBsAg, 4 (27%) also had HBeAg. Evidence of past HBV infection (presence of anti-HBc) was found in 34 (81%).

A total of 512 male and 111 female banana workers was identified. Of these 623, 290 (46%) were studied and blood was tested from 284. In the subgroup of 290 workers, the mean age was 32 years (SD=13); 228 (79%) were males. There was no indication of recent HAV infection, but 277 (97%) had anti-HAV, indicating prior infection. Twelve workers (4%) had anti-HBc IgM and 50 (18%) had HBsAg. Among those with HBsAg, 13 (26%) also had HBeAg. The majority of workers (219,

anti-HEV could be demonstrated in only 2 cases and it was not possible to distinguish between recent and past infection. The study of sera from Cowpen for evidence of HEV infection is continuing because ELISA tests for anti-HEV are still in the early stages of development (GOLDSMITH *et al.*, 1992; SKIDMORE *et al.*, 1992); sensitivity and specificity have not been completely determined. Work is under way to identify any antigenic variation among hepatitis E viruses. Because we could not establish a serological diagnosis for most of our hepatitis cases, these might have been due to HEV or another agent of non-A, non-B hepatitis.

In the absence of a demonstrable viral agent, consideration must be given to toxic substances, including ethanol or a contaminated food supply. Alcohol consumption was noted but collection of these data was outside the scope of the present study. There was no indication of disruption in the food supply during the dry season to suggest that hungry people might have consumed grains or other foods heavily contaminated with fungi and fungal toxins. Epidemiological analysis of a vast amount of demographic data collected for people studied is in progress, with a wide range of possible aetiologies being considered.

HAV and HEV have faecal-oral routes of transmission and problems existed with both drinking water quality and sanitation in Cowpen. Our study indicated that hepatitis A in Cowpen is an illness of children. HEV may have been recently introduced, thus explaining at least some of the cases of non-A, non-B hepatitis in young adults. Prevention of both HAV and HEV infections is strongly dependent upon a purified water source, good personal hygiene, and proper disposal of human waste. After attention was directed toward the probability of enterically transmitted hepatitis virus or viruses in Cowpen, some farm managers and local inhabitants built improved water systems that included treatment measures.

In screening refugees to the USA from Asia, Africa and eastern Europe, investigators have reported HBsAg prevalences ranging from 1.1% to 15.5% (CRYSEL *et al.*, 1991). Along the southern border of Mexico, another study reported the seroprevalence of HBsAg to range from 4.2% to 17.3%. This prevalence is uniquely high among the reported prevalences from the Caribbean nations. Overall, 91% of that Mexican population had been infected with HBV, and HDV was present in 50% of those who were HBsAg positive (ALVAREZ-MUNOZ *et al.*, 1989).

Within Central America, the lowest reported prevalence of HBsAg (0.64%) was found in Costa Rica (SALOM *et al.*, 1990). Throughout the Americas, from 1975 to 1985, HBsAg prevalence was reported to range from 0.3% in the USA to 8.0% in Amazonia. The countries surrounding Belize have a reported HBsAg prevalence between 1% and 3% (FAY *et al.*, 1990). However, the true impact and prevalence of viral hepatitis throughout many countries in Central and south America have yet to be studied (PAHO, 1990; WHO TECHNICAL ADVISORY GROUP ON VIRAL HEPATITIS, 1988). Cowpen HBV marker prevalences match or exceed the highest 1980 prevalences reported in Senegal, Thailand, Uganda, Egypt, and India (SOBESLAVSKY, 1980).

In the Cowpen population, with a very high prevalence of HBV markers, we found little evidence of HCV infection. HBV and HCV are transmitted through direct contact with blood, blood products and possibly other body fluids. However, infection with HCV appears to occur independently from HBV and is a frequent complication of transfusions (HAYASHI *et al.*, 1991). HBV can be acquired as a result of intravenous drug use with shared needles, blood transfusion and sexual contact. Individuals infected with HBV who are positive for HBeAg are considered highly infectious. HBeAg was found in 26%, 27%, and 54% of those HBsAg positive within the three populations we studied.

The specific risk factors that made the Cowpen population particularly prone to HBV infection are still under study. We do have information that parenteral antimalarials, vitamins, antibiotics and intravenous fluids were used without a physician's supervision, and that dental practices occurred outside a professional setting. Overall, people had a high level of interest in their health and the health of their family and friends. Generally recognized measures for preventing HBV infection include passive/active immunizations and counselling for behavioural modification. Efforts were initiated in 1991 in Belize to enhance the screening of blood donations for hepatitis markers. Screening of pregnant women for HBV infection must also be emphasized.

The high prevalence of HBV markers in the Cowpen population is also cause for concern about the potential for transmission of other blood-borne pathogens such as human immunodeficiency virus (HIV) and other retroviruses. Although human T cell lymphotropic viruses 1 and 2 have been found in 9% of one Indian tribe in Panama, it is not clear how transmission occurred (REEVES *et al.*, 1990). HIV infection is devastating the population in many of the same countries that reported high HBV rates 10 years ago. Greater knowledge about transmission characteristics is critical to the development of preventive

measures against such agents.

Our study emphasizes the need to learn more about the transmission of hepatitis viruses in rural Belize in order to institute specific intervention efforts. It is essential to determine when and where infection occurred since the banana workers of Cowpen were not a stable population. Most people studied came from surrounding countries and lived in Belize for variable periods. Additionally, our cross-sectional study was done when there may have been an increase of hepatitis in the region. We may have looked at a rate of infection which reflected exposure in Belize, outside Belize, or both.

In summary, we found a high prevalence of HBV infection markers and an unusually high prevalence of HBsAg positivity in Cowpen, Belize. Additionally, almost all people had evidence of HAV infection by the time adulthood was reached. Both hepatitis A and B are preventable through well established environmental controls and personal behavioural changes. Detailed descriptive epidemiological studies followed by intervention initiatives are needed to reduce hepatitis virus transmission and the associated morbidity.

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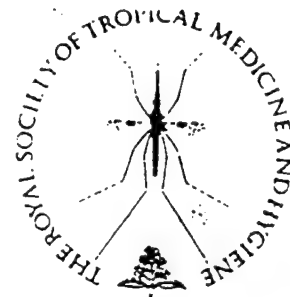
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**A CASE CONTROL STUDY OF RISK FACTORS
FOR LOW BIRTH WEIGHT OF INFANTS AT
BIRTH IN BELIZEAN POPULATION**

**M.T.M.H PROJECT IN BELIZE
BY
MOHAN KISHORE**

AUGUST 1992

A CASE CONTROL STUDY OF RISK FACTORS FOR LOW BIRTH WEIGHT OF INFANTS AT BIRTH IN BELIZEAN POPULATION.

BACKGROUND AND SIGNIFICANCE :

Low birth weight(LBW),defined as birth weight below 2500gms,is one of the most important maternal and child health problems in both developed and developing countries¹.The birth weight of an infant determines its chances of survival, growth and development. The birth weight is also an indicator of the health of the population of a country and is often considered as one of the highest health care priorities of a country¹. Birth weight is routinely measured for all deliveries in health care facilities.In developing countries average birth weight is lower than that reported for developed nations. Therefore the rate of neonatal mortality which is attributed to low birth weight is always significantly higher in developing countries. Neonatal mortality is preventable to a certain extent by good prenatal care.

The consequences of low birth weight are both short term and long term². About 100 years ago, a pregnant women's health care was provided by midwives, traditional birth attendants and family physicians. Today technology and medicine allow better prenatal care, although this is true more in developed than developing countries. Today the concept of prenatal care consists of clinical, social,

educational, cultural and psychological services. It is estimated that out of 122 million live births world wide in 1979, nearly 21 million had birth weight equal to or below 2500 grams^{1,2}. At the global level this means that about one in every 6 infants has a low birth weight, but the incidence is not evenly spread around the globe. In some parts of Asia the ratio of low birth weight is almost 1 in 2, while in parts of Europe it is only 1 in 17. Low birth weights ranges to 20% for Asia, 15% in Africa, 11% in Latin America, 8% in Europe, and 7% in North America. Of the low birth weight infants born in 1979, about 19 million (90%) were born in developing countries¹. The mean birth weight in Asia for that year was about 2900 grams, in Africa, 3000 grams, in Latin America, 3100 grams, in Europe, 3200 grams, and in North America, 3200 grams. Studies show that the number of prenatal clinic visits, age of the mother, educational status, socio-economic conditions, cultural and ethnic background, and personal habits contribute to low birth weight³⁻¹⁰. The rate of low birth weight in 1984 for USA was 6.7% ; the range was from 4.8% in Alaska to 12.5% for the District Columbia ¹¹.

Belize, a Central American country, has a population of 180,000 people. Belize has a number of ethnic groups with varying health care practices. About 80% of the population attends primary school education. A majority of pregnant women attend prenatal care. In addition Traditional birth attendants provide prenatal care. In one of these ethnic groups for example, the husband delivers the baby, and most of the mothers in this group do not utilize health care facilities. Belize reports one of the

lowest infant mortality rate in the region (19.9/1000 live births), and the maternal and child health care programs in most parts of the country are well organized by the ministry of health. Earlier studies have shown that 10% of newborns in Belize have low birth weight¹². A case control study was designed to determine possible risk factors attributed to low birth weight. The following were the aims of the study.

SPECIFIC AIM: To investigate whether there are associations between low infant birth weight and other maternal characteristics in a Belizean population.

The hypotheses are: (1) An infant's birth weight will vary according to (a) number of prenatal visits of its mother and (b) which trimester of pregnancy prenatal care was initiated.

(2) There is an association between an infant's birth weight and maternal age at infant's birth.

(3) There is an association between an infant's birth weight and maternal nutritional status as measured by hemoglobin level.

(4) There is an association between an infant's birth weight and the mother's parity.

MATERIALS AND METHODS.

Study population :

This case control study that was based on the existing data from the maternity ward, nursery, the Medical statistics department and the records office at Belize city

Hospital. Permission to conduct the study was obtained from the Director of Health Services, ministry of Health, Belize. Belize city has two prenatal clinic facilities and one General Hospital for delivery and other services operated by Ministry of Health. an average about 2000-2500 deliveries a year occurred at the Belize city hospital. Cases and control infants were selected from all those delivered during year 1991 at Belize city hospital.

Selection of cases: Belize City hospital's maternity ward maintains a delivery registration book. All the deliveries that were registered every day with infant's information. All the children born between January 1, 1991, to December 31, 1991, inclusively were chosen as cases with birth weight of baby less or equal to 5.5 pounds(2500 grams). The other criteria for the cases was singleton delivery. About 184 cases were registered in the study, although the sample required for the study were only 165.

Selection of Control: Controls had the same criteria like the cases with the exception of birth weight. The required birth weight was more than 5.5 pounds(2500 grams).

Sampling Method : The total number of deliveries were 2300 for 1991 year. About 184 cases were registered in the study, although the required sample size for cases was only 165. The controls were selected from the remaining 2116 births. A systematic sampling method was utilized in which every third baby from the birth registration book was selected(excluding cases). A sample of 506 were selected although the required sample were 495.

Cases and controls were compared for the maternal demographic profile and maternal risk factors.

RESULTS: Table 1. shows the mean age of the mother in the study was 23.29 +/- 5.6 and 24.39 +/- 5.83 for the cases and control groups respectively. The two groups were not significantly different in terms of age ($p = 0.6133$). Race of mother in two groups were significant, particularly in Spanish and Mayan groups ($p = 0.00099$). The other two groups of race, Creole and Garifuna were not significant statistically. The family income showed significance ($p = 0.0343$) in both groups especially in low and middle income groups. Maternal education had no significant among both groups ($p = 0.1673$) and the majority of pregnant women had primary education. Parity showed no significance in case and control group ($p = 0.6758$). Marital status was also not significant in two groups ($p = 0.5179$). Maternal weight showed significance among cases and low maternal weight has contributed low birth weight babies ($p = 0.0135$). The sex of the baby ($p = 0.0107$) showed significance in both groups.

Table 2. shows the demographic characteristics with the odds ratio and the confidence intervals among cases and controls. Spanish race had the highest percentage of control group infants and it signifies the better outcome. The Mayan race had higher percentage of low birth babies and overall the group has to be motivated for better outcome in future. The Creole race was among the least low birth weight babies (OR = 0.62), and the Mayan race were 3.39 times more likely to contribute low birth weight babies. The lower and middle income groups had a

significant low birth weight outcome than higher income groups. The odds ratio among parity, marital status, maternal education were not significant.

Table 3 and 4 shows the possible etiologic factors that contribute low birth weight in case and control groups. Prenatal visits had significance in both groups ($p=0.00002$). Mothers with less than three prenatal visits were 2.59 times more likely to produce low birth weight babies ($OR = 2.53$). Gestational age has no significance among both groups. Maternal hemoglobin had significance among both groups ($p=0.0005$). There was significance among pregnancy complications ($p=0.0000$) such as preeclampsia and breech presentation. These complications were 1.17 times more likely to cause low birth weight babies. Past miscarriages had 19.97 times more likely to contribute low birth weight babies ($OR = 19.97$). There was also great significance among two groups in occurrence of medical conditions such as Sickle cell disease, diabetes and urinary tract infections ($p=0.0019$). The infections were 8.49 times more likely to contribute low birth weight babies.

CONCLUSIONS: Risk factors associated with low birth weight were low family income, low frequency of prenatal visits, and maternal health status (anemia, hypertension, medical conditions). Although there is no easy interventions to increase family income, increasing the number of prenatal visits and treatment of maternal medical problems such as anemia, hypertension can be improved. This might best be accomplished by establishing or enhancing the resources of a high risk maternity clinic at Belize City hospital. High risk pregnant women living outside of Belize City should be provided with government assistance to allow them to be followed at regular

intervals in this clinic. The financial benefit of this type of prenatal care in high risk pregnancies expected to be cost-effective.

Good antenatal care includes an early prenatal visit in first trimester, frequent visits in second and third trimester, recommended in the schedule of Primary Health Care of Belize¹². Common causes of anemia in women of child bearing age in Belize, in decreasing order of frequency are malaria, hookworm infection, malnutrition, and repeated pregnancies or unplanned pregnancies. Most of the causes of maternal anemia are preventable. Good nutritional food habits can be motivated by health education. Good primary health care in pregnancy should help to reduce the prevalence of anemia among pregnant women and other causes of premature birth.

Mayan population has to be mobilized to reduce the low birth weight babies. These people were generally not utilizing the health care services. The number of prenatal visits has to be increased to reduce the low birth weight. More focus has to be given to reduce pregnancy complications, past miscarriages and to prevent pregnancy complications.

RECOMMENDATIONS: (1) Strengthen health education/ information for women of child bearing age in Belize. This should emphasize prenatal attendances, adequate nutrition and child spacing.

(2) Strengthen primary health Care services to prevent, manage maternal conditions, especially preeclampsia, placenta Previa and other late complications of pregnancy.

Table 1. Demographic characteristics of case control study of low Birth weight infants in Belize.

Variables	Cases N=184 (%)	Controls N=506 (%)	P-value
Age of Mother			
Mean age	23.29	24.39	0.613270
S.D +/-	5.6	5.83	
Race of mother			
Creole	107 (58.2)	285 (57.9)	0.000991 *
Spanish	32 (17.4)	137 (27.1)	
Garifuna	22 (12.0)	54 (10.7)	
Mayan	14 (7.6)	11 (2.2)	
Others	9 (4.9)	19 (3.8)	
Family Income(US \$)			
2400	11 (6.0)	28 (5.5)	0.034392 *
4800-7199	140 (76.1)	332 (65.6)	
> or =7200	33 (17.9)	145 (28.7)	
Maternal Education			
1-8 yrs	126 (68.5)	344 (68.0)	0.167260
9 or > yrs	50 (27.2)	111 (21.9)	
Unknown	8 (4.3)	40 (7.9)	
Parity			
Primi	63 (34.2)	166 (32.8)	0.675800
2-3	70 (38.1)	185 (36.6)	
>3	51 (27.7)	155 (69.4)	
Marital Status			
Married	34 (18.5)	119 (23.5)	0.517860
Unmarried	80 (43.5)	210 (41.5)	
Common-law	32 (17.4)	93 (18.4)	
Unknown	38 (20.7)	83 (16.4)	
Maternal Weight			
Mean	132.64	140.94	0.013499 *
S.D +/-	25.94	47.41	
Sex of the baby			
Male	81 (44.0)	258 (51.0)	0.010668
Female	103 (55.9)	248 (49.0)	
Birth Weight of baby			
Mean	4.73	7.43	0.000000
S.D +/-	1.01	1.12	

* Significantly different.

Table 2. Demographic characteristics of case control study of low Birth weight infants in Belize.

Variables	Cases N=184 (%)	Controls N=506 (%)	OR (95% CI)
Age of Mother			
Mean age	23.29	24.39	
S.D +/-	5.6	5.83	
Race of mother			
Creole	107	285	Ref
Spanish	32	137	0.62 (0.39-0.99)
Garifuna	22	54	1.09 (0.61-1.93)
Mayan	14	11	3.39 (1.40-8.29)
Others	9	19	1.26 (0.51-3.05)
Family Income(US \$)			
2400	11	28	1.73 (0.72-4.07)
4800-7199	140	332	1.85 (1.14-2.24)
> or =7200	33	145	Ref.
Maternal Education			
1-8 yrs	126	344	0.81 (0.54-1.23)
9 or > yrs	50	111	Ref.
Unknown	8	40	
Parity			
Primi	63	166	1.15 (0.73-1.81)
2-3	70	185	1.15 (0.74-1.79)
>3	51	155	Ref.
Marital Status			
Married	34	119	Ref.
Unmarried	80	210	1.33 (0.82-2.17)
Common-law	32	93	1.20 (0.67-2.17)
Unknown	38	83	
Maternal Weight			
Mean	132.64	140.94	
S.D +/-	25.94	47.41	
Sex of the baby			
Male	81	258	0.76
Female	103	248	Ref.
Birth Weight of baby			
Mean	4.73	7.43	
S.D +/-	1.01	1.12	

Table 3. Possible Etiologic Factors for Low Birth Weight.

Variable	Cases N=184 (%)	Controls N=506 (%)	P-value
Prenatal Visits			
< 4	44 (10.4)	56 (11.1)	0.000022 *
4 +	140 (89.6)	450 (98.9)	
Gestational age at first visit			
1st Trimester	51 (27.5)	137 (27.2)	0.945331
2nd Trimester	94 (52.1)	267 (52.6)	
3rd Trimester	39 (20.4)	102 (20.2)	
Maternal Hemoglobin			
Mean	11.02	11.25	0.000556 *
S.D +/-	1.11	1.09	
Pregnancy complications			
Preeclampsia	7 (3.8)	1 (0.2)	0.000000 *
Placenta previa	1 (0.5)	0	
Breech	12 (6.5)	2 (0.4)	
others	0	2 (0.4)	
Past Miscarriages			
Present	7 (3.8)	1 (0.2)	0.463512
Absent	177 (96.2)	505 (99.8)	
Medical conditions during pregnancy			
Present	178 (96.7)	504 (99.6)	0.001940 *
Absent	6 (3.2)	2 (0.4)	
Hypertension (Diastolic Blood Pressure >90 mm Hg)			
	16 (0.08)	53 (0.10)	0.028908 *

* Significantly different.

Table 4. Possible Etiologic Factors for Low Birth Weight.

Variable	Cases N=184 (%)	Controls N=506 (%)	OR (95% CI)
Prenatal Visits			
< 4	44	56	2.53 (1.59-4.0)
4 +	140	450	
Gestational age at first visit			
1st Trimester	51	137	Ref.
2nd Trimester	94	267	0.95 (0.62-1.44)
3rd Trimester	39	102	1.03 (0.61-1.72)
Maternal Hemoglobin			
Mean	11.02	11.25	
S.D +/-	1.11	1.09	
Pregnancy complications			
Preeclampsia	7	1	1.17 (0.86-39.62)
Placenta previa	1	0	
Breech	12	2	Ref.
others	0	2	
Past Miscarriages			
Present	7	1	19.97 (2.46-439.9)
Absent	177	505	Ref.
Medical conditions during pregnancy			
Present	6	2	8.49 (1.54-61.38)
Absent	178	504	
Hypertension (Diastolic Blood Pressure >90 mm Hg)			
	16	53	

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AIDS/ HIV in BELIZE

INDEPENDENT MPH PROJECT

**SUBMITTED BY
MICHAEL PITTS
AUGUST '92**

MPH INDEPENDENT PROJECT:

OBJECTIVE 1: UPDATE ON HIV/AIDS IN BELIZE

INTRODUCTION:

Since AIDS was first diagnosed in 1981¹, the number of individuals affected has increased at an alarming rate, moving quickly to the now established full blown AIDS pandemic. Initially this disease was reported as affecting homosexuals and intravenous drug users in the USA and HAITIAN heterosexuals². With the rapid changes, overlapping life styles, revolutionized sexuality, increased migration and cultural exchanges, AIDS has moved beyond those initial borders and virtually any one can fall victim to this disease. Since DR Gallo and his French counterpart implicated the Human Immunodeficiency Virus in the late 1970'S to 1981(1), several tests to diagnose this disease have been developed. There is the ELISA test(an antibody test) used for screening and the WESTERN BLOT test used for confirmation. Recently there was further progress to establish a cheap, quick antigen test which will pick up the disease at its inception and remove that critical lag period that occurs between getting the virus and developing antibodies³. A further benefit of this approach is to make way for the development of vaccines which could be administered in antibody form in a similar fashion to what is done for HEPATITIS B. As the cure of this disease remains elusive, it is imperative that countries take stock of the status of this scourge.

The current wisdom of WHO is that there must be international surveillance and cooperation in the control of this disease. Since 1987, national surveillance has been spreading from country to country and there is statistics reported biannually for 177 countries worldwide by WHO. This reporting is of AIDS and experts from WHO estimate that there is under reporting which ranges from 90 % in parts of AFRICA to 20 % in the U.S⁴. So far there has not been international HIV reporting. This seems to be due to lack of resolve, concern for confidentiality of patients, fear of

discrimination to those who are positive, and the fact that there is no cure for this disease. This trend is slowly changing as various therapeutic and prophylactic modalities are developed to treat some of the opportunistic diseases that occur with HIV infection and also the development of drugs, for example AZT, that delay the appearance of AIDS (2 & 3). It seems prudent that, as the cure for AIDS is sought, there would be complete or at least some better level of HIV surveillance internationally to prepare for the future. This process will follow the pattern that exist for AIDS, reinforced by appropriate legislation.

WORLD SCENARIO:

As of MARCH 1990 there were 237,110 cases of AIDS reported worldwide. From the Americas there were 150,619 cases, USA alone accounted for 124,282 of those cases. Brazil was next in the Americas with 10,058 cases. Africa accounted for 51,978, while Europe reported 31,948 cases. Oceania reported 1,947 cases with the total of Asia reporting the remaining 618 cases for this period. These results were based on reporting rates that ranged from 90 % under-reporting in Africa to 20 % in the U.S. When these figures are analyzed in terms of cases per 1000 population the Americas was leading with a rate of 0.2072 cases/1000. Oceania was second with 0.0749, with the other regions in the following order: Africa = 0.0654, Europe = 0.0404, and Asia with 0.0002. These rates when adjusted for the different levels of reporting may show a different order.⁵

REGIONAL SCENARIO - CENTRAL AMERICA AND CARIBBEAN

Between seven states of Central America there were 458 AIDS cases reported by 1989 and this increased to 717 by the time of 1990 report. The following list indicates the trend in those countries over the reporting period. The cumulative numbers of cases when analyzed in cases/1000 population showed Honduras to be the front runner with a rate of 0.117 and Panama second with a rate of 0.079. Belize, which has the smallest population in Central

America, ran a close third with 0.061 cases/1000 population. Nicaragua had the lowest with a rate of 0.001, lower than that seen in Cuba (0.0063) in the neighboring Caribbean. See attached charts (1,2 ,7-11).

From 20 countries of the Caribbean there were 3240 cases reported in 1989, and by 1990 there were 5,232 cases of AIDS. From thirteen countries reviewed in the Caribbean, Bahamas was number one with a rate of 1.852/1000 population. Trinidad and Tobago, and Barbados ran distant second and third with rates of 0.464 and 0.441 respectively. Haiti, which was notorious for AIDS in the early 80's, was fourth with 0.423. Jamaica was twelveth with a rate of 0.059, lower than for Belize(0.061). Cuba was last with a rate of 0.0063, ten times less than Belize. These figures must be viewed as the best guides in the AIDS trend and will suffice until better surveillance and more complete reporting is in place(5). See attached charts 3-6.

B: BELIZEAN SCENARIO -

Belize is a developing country in central america with a small economy which is based on agriculture and tourism. Of a national budget of around 200 million Belize dollars, 5 to 7% is set aside for health care⁶. As it stands this is barely sufficient for the country to manage the traditional health problems. Now, like other neighboring countries, it is faced with the HIV and AIDS epidemic. The numbers have placed this country in staggering prominence due to high percentage of cases with the potential of stressing the health budget if the disease is allowed to continue unharnessed. So far what seems to be protecting the society is the smallness of the population and the relative ease with which cases potentially could be contacted, isolated and followed. The structure of the health system is quite fragile in its ability to cope with a full blown epidemic, a daring and sinister problem. Thus far the problem has been dealt with through the diagnostic and therapeutic efforts of a single nurse/venereologist, operating out of a small loosely organized and funded clinic. To date, apart from a meager

subvention from a contracted national health budget, this clinic's venture is run with assistance from British medic corps, Agency for International Development (US), some funds from European Community, and some other benevolent organization. So far there is no single unit in the Belize health system that can deal with the HIV/AIDS patient through the complete spectrum of his illness. There are few health providers prepared to deal with these patients. Lastly, diagnosing ,reporting , follow up and counselling is at best a loose arrangement⁷.

There are some key cultural and societal trends that put Belize at risk for HIV. There is a growing homosexual community which has not been operating in the open since the community is not quite tolerant of this 'deviant ' activity. Then there is the institutionalized habit of casual sex reinforced by the multi-partner activity of both male and females. The population is 50% Roman catholic and, as such, is less inclined to entertain use of condoms in any anti-HIV campaign. For condom to be effective against HIV transmission, it must be used by an individual more than 90 % of the time⁸. Also factoring in the equation is a current growing drug culture. This involves use of crack and other forms of cocaine and marijuana. Even though IV drug use is at a minimum, what puts the community at risk is that addicts are involved in many forms of unsafe sex in order to get drugs. Another cultural feature is that in some groups when a young man is reaching of age, he will engage in sex with prostitutes. These prostitutes may be locals, but more often are foreign latinos working in Belize or encountered in brothels across the border in Guatemala, Honduras or Mexico. This activity may bring this group in contact with a likely reservoir of HIV. Finally, there is a significant group of young Belizean who go to the USA and become involved in high risk activity. A number of young Belizean males reach the USA and become involved in the illegal drug trade. Either on the streets or when they are in jail in the USA, they are in contact with high risk people ,through drug use, casual sex or homosexual activity which could be consensual or forced. There is also the practice that

Belizean who test positive with HIV are soon repatriated to Belize.(7)

C: OVERVIEW OF HIV/AIDS FROM CURRENT DATA IN BELIZE :

Currently sexually transmitted diseases are dealt with by a public health nurse and aids coordinator through one central clinic in Belize city. The nurse has long term practical experience in diagnosis , treatment, counselling, and follow-up reinforced by numerous seminars and training programs. The AIDS coordinator was seconded from the British army after his tour of duty as their STD health worker came to an end. The data on the present AIDS problem in Belize have been assembled by these two individuals. AIDS cases are picked up through clinical diagnosis followed by laboratory confirmation. HIV cases are picked up through routine testing of patients with other STD's or from screening via blood collection drives. The tests done are structured with combination of ELISA screen followed with western blot confirmation. Western blot confirmation is obtained through cooperative efforts with USUHS and CAREC laboratories. There is general reporting from governmental and non-governmental agencies to a central unit. It is estimated that there is at least 60 % under reporting with significant duplication. The true levels will not be certain until clearer surveillance of HIV/AIDS is accomplished. This may be accomplished after a serious national resolve and a considerable outlay of financial and man power resources. Any financial burden and intrusion of privacy due to improved surveillance may well be worth having in order to better prepare and protect the nation from this raging epidemic. By picking up HIV positives earlier, programs to limit its spread may be more effective. Also, use of drugs like AZT could have a better chance to prevent the development of full blown AIDS and also the blood supply could become safer by this type of intelligence gathering as it were. I think the Cuban model may well be the wave of the future . This model has gotten beyond the

hysteria and has returned some of the practices that were used in the campaigns against tuberculosis and leprosy before cure for these were discovered. Both these diseases were seen to be less contagious than HIV.

CURRENT SURVEILLANCE REPORTING OF HIV/AIDS

From data compiled by the chief venereologist in Belize (Jenkins and Carr), there are presently 154 cases of HIV in Belize. This by no means represents the complete picture as there may be as high as 60-70 % under reporting or undetected cases. Forty seven of these have converted to full blown AIDS of which 42 have died to date. The index case was a female in 20-30 age group. She came from USA, where she engaged in casual and promiscuous sexual activity, to Belize in 1987. She died in the southern town of Dangriga in 1989. This southern town later became one of the leading areas of incidence and controversy pertaining to AIDS in Belize. Since then the focus of HIV/AIDS has shifted to Belize and Orange Walk district. Present data shows that the age group mostly affected is in 20-40. There are four cases of vertical transmission, with the youngest HIV infected person being 18 months. The following graphical presentations show the trend that has occurred in the reported cases. These were made available through the courtesy of the medical statistical office of Belize. SEE ATTACHED CHARTS.

From chart 12, of the 43 cases of AIDS reported, 49% was transmitted by heterosexual contact. Homosexual and bisexual contacts were implicated in 19% and 14% of cases respectively. 7% of AIDS were perinatally contracted, while 2% was by blood transfusion. There is a remaining 9% of cases for which the mode of transmission was not determined. The predominance of heterosexual mode of transmission of AIDS in Belize is similar to what is found in Africa, Jamaica, and in parts of the US. Over the six districts of Belize, most of the AIDS (25) and HIV (74) cases were reported in Belize district where a third of the Country's population lives.

Cayo and Stann Creek were quite close in both AIDS and HIV cases. The former reported 25 & 6 while the latter reported 23 HIV and 5 AIDS cases respectively. Orange Walk was third in number of HIV reported (13), while Toledo reported 4 cases of AIDS⁹. See chart

(1 3)

From a racial stand point it seems the Creole, Latino and Garifuna's are the groups most affected by this disease. So far, there were no cases reported in traditional Mayan, Chinese, and Mennonite populations. It could well be that they are missed. The wisdom held is that these three groups are spared because of being relatively closed, holding strong family values with great sanctions on casual sex and low rates of promiscuity. This was the view of the venereologist in Belize.

Males account for 73.8% (31) of AIDS and 68.9% (91) of HIV cases. Females were 26.2% of AIDS and 31.9% of HIV positives. The age group 20-39 accounted for 73.8% of AIDS cases and 82.6% of HIV positives. The youngest AIDS case was 18 months and the oldest 60+ years(9). This may be important in later control of this disease as this group is the one that is sexually active and ought to be responsible for their sexual acts. It is also noteworthy that this group follows the late pubertal period when a number of teenagers are experimenting sexually. See charts.

Review of reported gonorrhoea and syphilis cases show that these are on the increase since 1989 in Belize. Males were infected more than females on every instance(9). The importance of this report is that having other STD's increases the likelihood of contracting HIV and AIDS. Men may be at higher risk of HIV and this may be the source of control of this disease through use of condoms or controlled sexual behavior in the Belize population. Further reports from the central clinic have suggested that for each female HIV positive there is at least two contacts. For the males, there are at least five contacts. So far of the 154 HIV positives in Belize 47 have converted to AIDS and the reported average life

expectancy is between 15- 30 months. Forty one of the AIDS cases have died so far. There are reports of multiple drug resistant tuberculosis occurring, but patients mostly suffer gastrointestinal, pulmonary or urinary tract opportunistic diseases. Candida stomatitis is a frequent feature. Few cases of Kaposi sarcoma was reported. These diseases are not well documented in the Belize statistics and further study is warranted(7).

The travelling patterns of the HIV /AIDS cases in Belize is not well documented. However, what is of particular interest is the practice of Belizean males in the high risk age group to visit brothels in Guatemala, Honduras and Mexico. Also, casual sexual activity with some foreigners of high risk behavior(drugs and liberal sex). Up to present, 3 cases from Guatemala have been imported, one male heterosexual and two female prostitutes. From Honduras, three females, one prostitute and two heterosexual women. Honduras has the highest rate of AIDS in Central America. The index case was a Belizean returning from the US after a long stay.(7)

From this review there could not be any hard held conclusion on the HIV/AIDS situation in Belize. For the statistics to be more meaningful, better surveillance of both AIDS and HIV needs to be instituted. With the necessary resolve and legislation in place better and more uniform data collection could be assembled. Then a more scientific analysis of the data could be done which would help in predicting the true status of this epidemic in Belize. Until then programs that are in place may be ineffective.

PART B:

Background and definitions:

The next five objectives were met in developing a surveillance program for Belize. Surveillance is the systematic regular collection of information on incidence or prevalence , distribution and trends of a specific disease in order to achieve effective control of that disease. There are general population and sentinel population surveillance. General population surveillance, while it is more complete, requires large amounts of manpower and resources

for planning and implementation. Because it samples many subgroups together, valuable information of individual groups maybe lost. In the case of AIDS/HIV, the information about varying subgroups is highly essential for adequate control, prevention and treatment. Furthermore, because of the stigmata, level of discrimination, and the nature of the disease, direct incidence of HIV is hard to obtain. Bearing these factors in mind, along with restraints of limited resources, a sentinel HIV prevalence surveillance is proposed. Sentinel surveillance allows for the sampling of different subgroups and prevalence study circumvents some of the problems that befalls incidence studies of HIV. It is important to remember that incidence can be determined indirectly from sequential prevalence studies on the same population. The type of surveillance program proposed to obtain reasonable HIV prevalence trends is a sentinel program that relies on unlinked anonymous testing. By this method, the individual that is being tested do not know when they are tested and the person doing the test do not know who they are testing. The end result is that no test can be linked to any individual and that result becomes a statistic with demographic markers of interest. This is further reinforced by randomness of testing and coding that masks identity. These statistics will be derived from test done on sera that would have been discarded after otherwise routine testing. The source of these sera will be sentinell sites, those place of interest where bloods are routinely taken for purposes other than HIV testing.

Determining sample size:

For the purpose of this type prevalence study the total number of samples taken from each site will be determined using epi info. Calculation for sample size will be made based on the size of population at site, the prevalence of HIV in the population at large, the margin at which it is expected the prevalence may vary and the confidence interval. Samples for the purpose of the survey will be obtained from sera remaining from routine blood test in the specific clinics. The samples will be chosen randomly each clinic day, using table of random numbers, until the required number is

obtained. Samples will undergo two stage ELISA test. Confirmatory test will be done using the Western blot method. The two stage ELISA testing is to allow for greater reliability in the screening process.

Choosing sentinel sites:

Sentinel testing by design allows for sampling small numbers of clients and specific groups. For this to be worthwhile in terms of HIV in Belize, the following sites will be the initial sentinels: Matron Robert's antenatal clinic, Belize defence force clinic, the sexually transmitted disease clinic in Belize city and the Belize city hospital blood bank. At the antenatal clinic chosen there are about 800 clients annually and by the epi info method described, the sample size will be 40. This is based on 0.08% prevalence, a margin of 0.8% and 90% confidence interval. The small interval is chosen because the population is small. The first three sites are very important in that they will allow sampling of the sexually active age group in the population. The last is important in that the blood bank is a very essential service in the health care system and it represents one of the major means of transmission of HIV. This sentinel surveillance will start in the Belize district and then duplicated in the other five districts if successful. It is expected that it becomes an annual process.

Choosing samples:

Samples will be obtained by using a table of random numbers. To be included as a sample that individual will need to be making the first clinic visit for that year. Persons with second visits will be excluded. If there is any situation where the identity of the individual is known and could be linked to a results they will be dropped from the sample pool.

Maintaining confidences:

To ensure confidentiality the samples will be coded by head of the sentinel program, documenting along with that sample pertinent demographic parameters. Laboratory request forms will carry code but devoid of demographic data so that lab technician will be able only to keep sample separate. Results will necessarily be kept

separately and access will be restricted. Data when released will be done so that test results can never be matched to any individual. The random number by which sample was chosen will be use for entering data for analysis.

Parameters of importance:

The parameters of interest to be noted in this sentinel program will be age group, sex, region of country from which sample comes , whether they are blood donors and presences or absence of stds. This information will be obtained from routine data that would have already been collected. More data than this is likely to allow linkage of results to individual.

Analysis of data:

Analysis will be done to describe HIV occurrence in terms of the parameters. Data will be reported in forms of frequency charts or pie charts. This data will be used as to set baseline levels and later to establish trends. Where there are interventions, this system will be able to assess the impact on HIV prevalence in the different groups. Finally all the results could be summed up together to approximate what would have been achieved from a general population survey.

Conclusion:

Even though positive results are expected, it is impossible to offer treatment since individuals would not be known. This limitation is intrinsic to the unlinked anonymous system proposed, allowing the 'ethical' issue of non-treatment to be avoided. Because this issue may be a pressing concern it may be advisable to offer parallel anonymous testing at some sites. In those instances pre-test counselling will be necessary and also provisions for post-test treatment and counselling.

References:

1. Aids/Hiv report 90
2. Aids/Hiv report 87
3. Journal of Clinical Microbiology, April 1990
4. WHO ,Aids/Hiv report, February 28, 1989.

AIDS SURVEILLANCE IN THE AMERICAS

Summary

Data as received by 10 March 1992

Cumulative number of cases reported

worldwide: 454,888

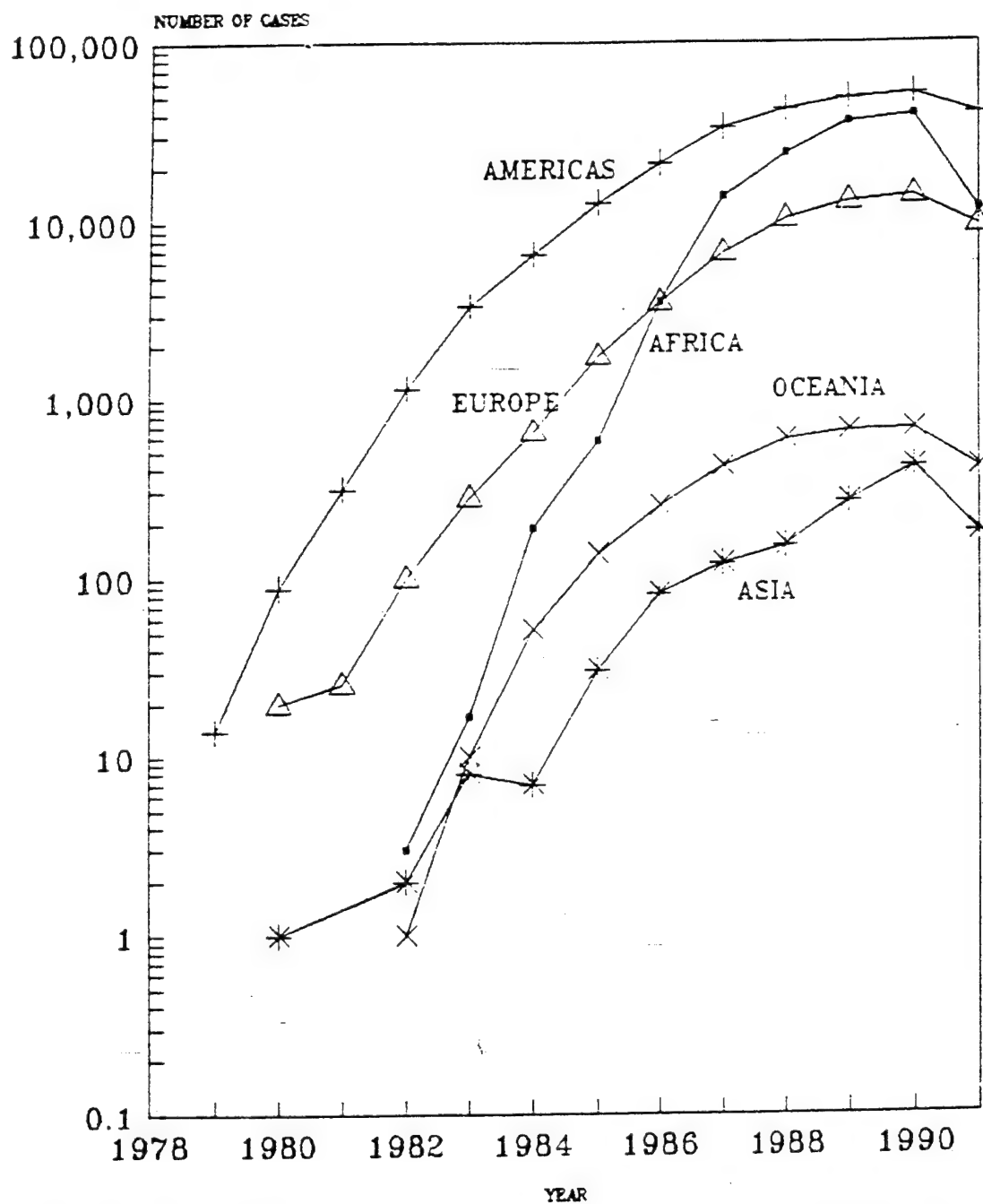
Cumulative number of cases reported

in the Americas: 261,184

Cumulative number of deaths reported

in the Americas: 158,625

Fig.1. REPORTED AIDS CASES, WORLDWIDE, BY YEAR,
BY REGION OF THE WHO, 1979-1991.*



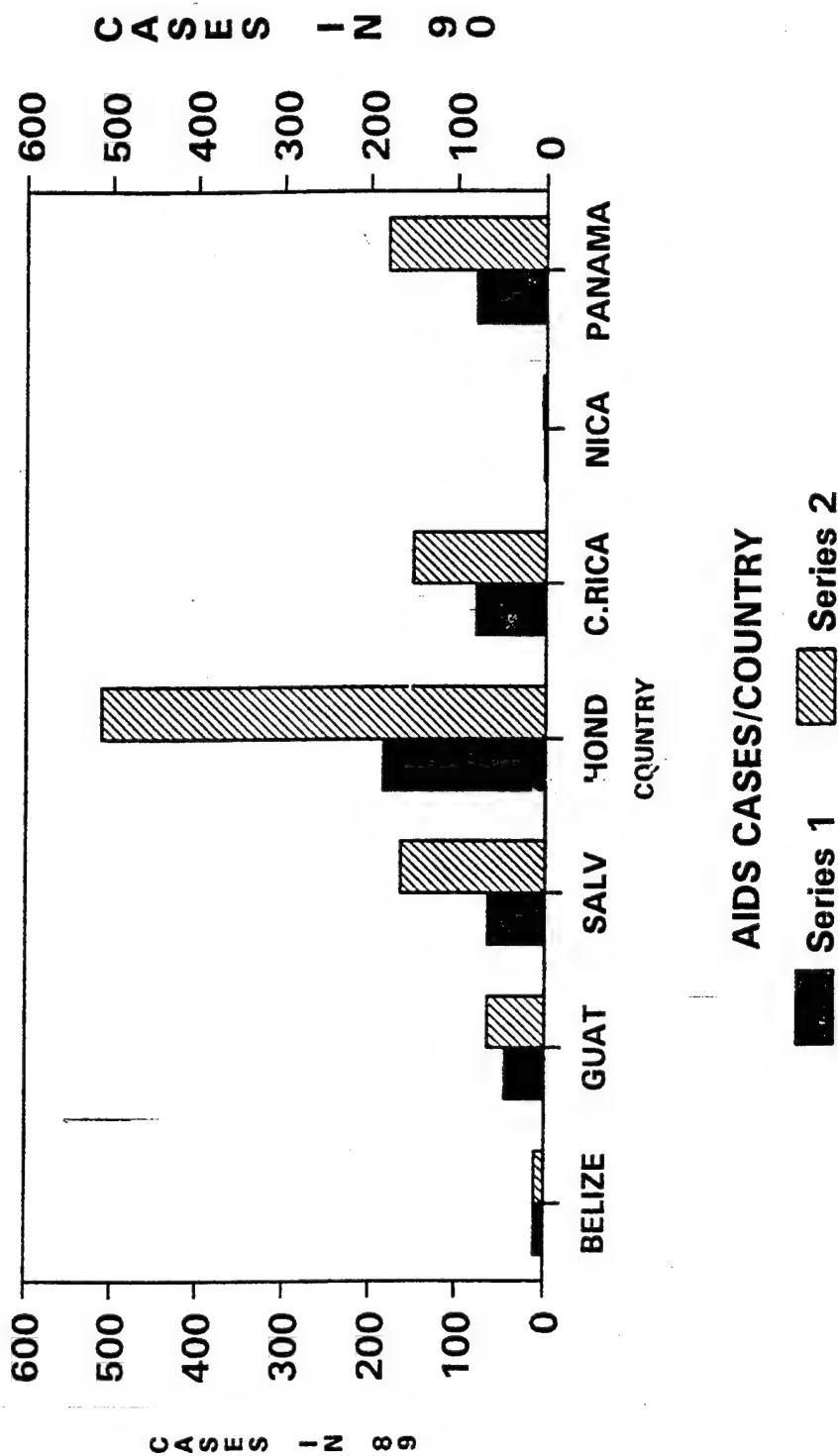
*DATA FOR 1991 ARE INCOMPLETE.

ENHGMAR92\WHOTRND5

Region of Americas # AIDS cases for 89 reported MARCH 90 + MAY 91

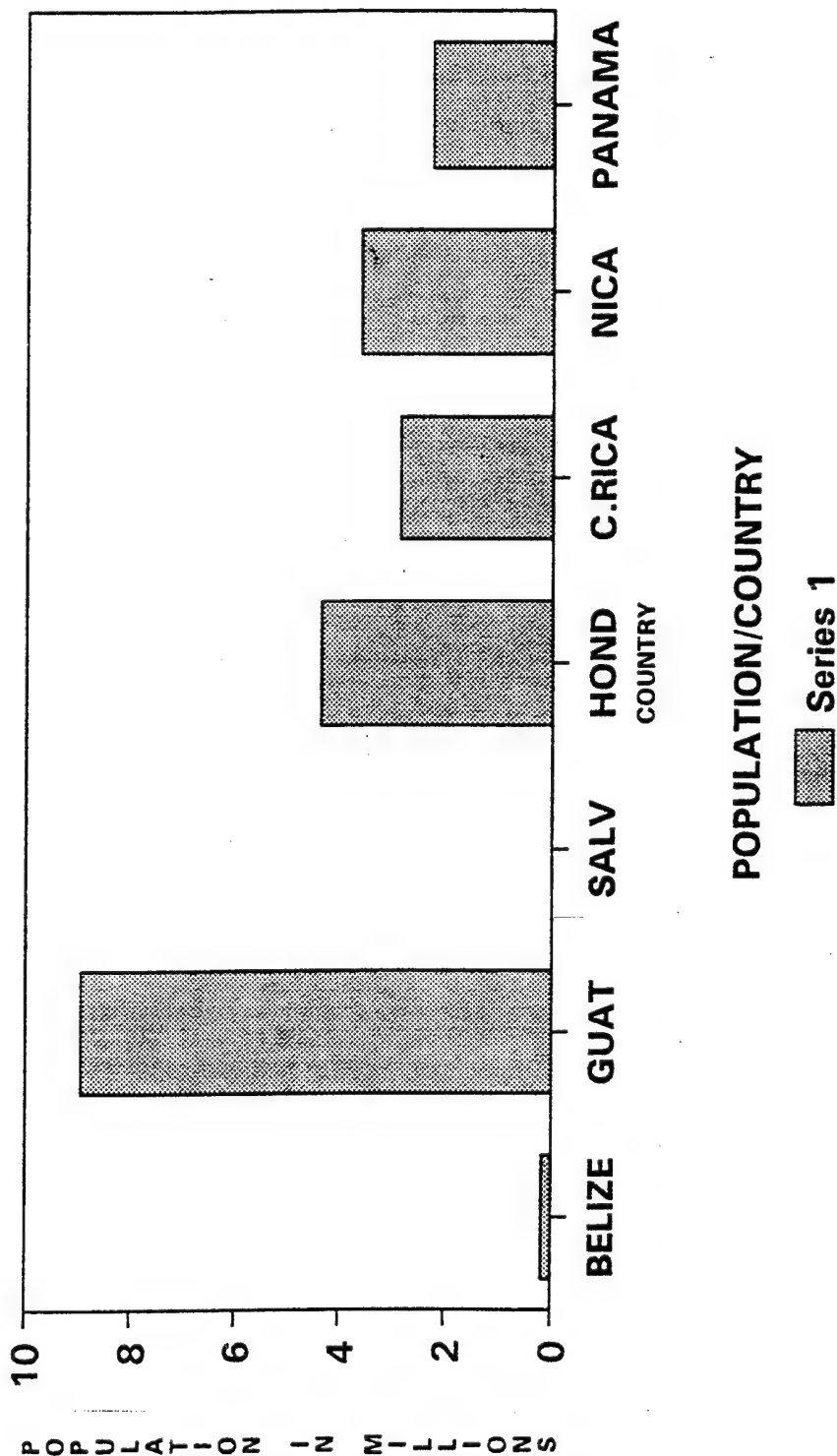
subregion	March 90	May 91
Andean	752	806
Southern cone	353	335
Brazil	3,706	4,516
Central Amer Isthmus	538	531
Mexico	1,434	1,499
Latin Carib	839	994
Caribbean	631	725
North America	28,880	37,517

AIDS CASES 1989-90 REPORTED IN CENTRAL AMERICA



SOURCE:WHO AIDS/HIV REPORT

AIDS CASES 1989-90 REPORTED IN CENTRAL AMERICA



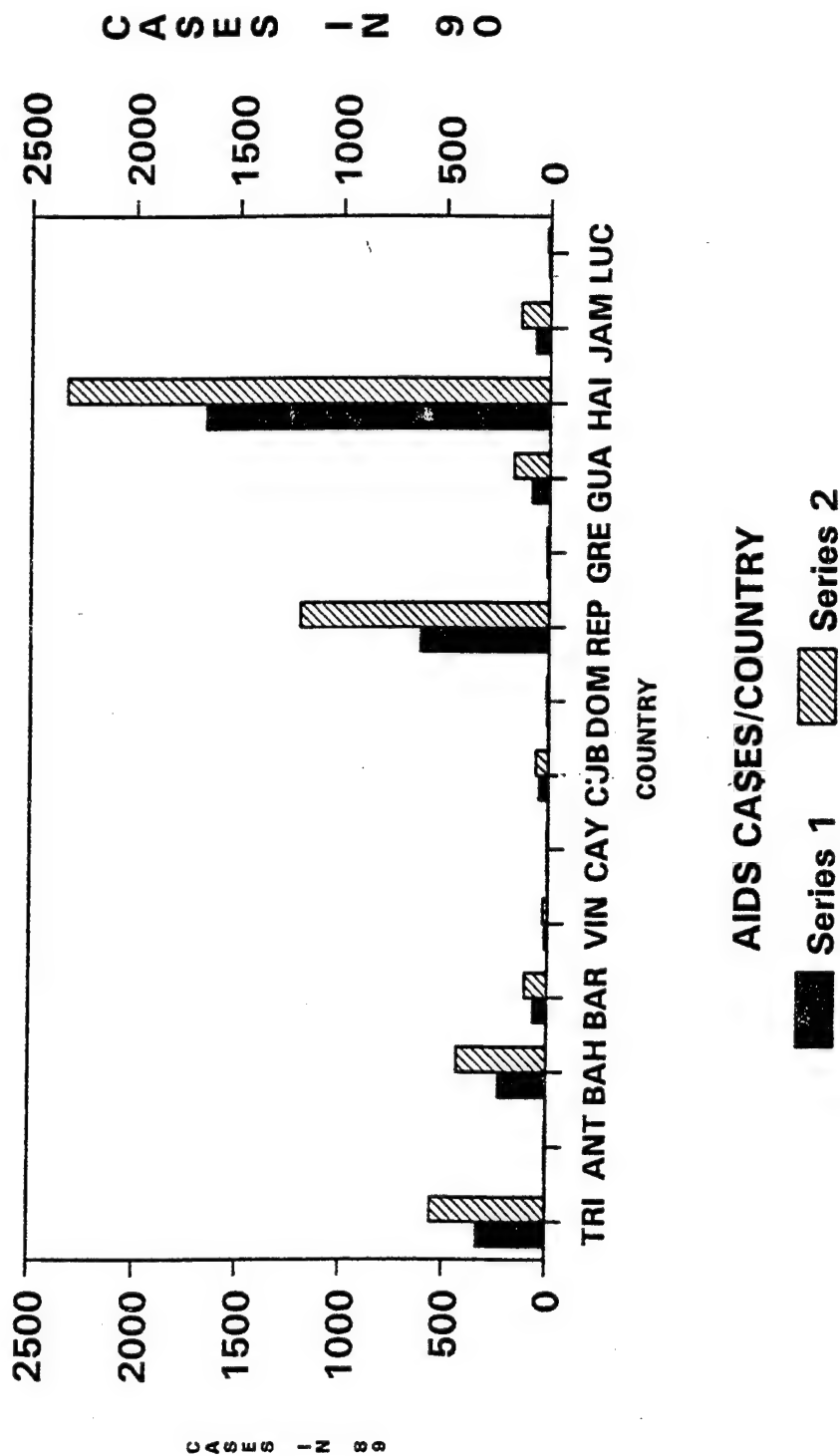
SOURCE:PAHO REPORT 92

AIDS CASES PER 1000 POPULATION

FOR CENTRAL AMERICA 90

COUNTRY	CASES /1000 POP
BELIZE	0.061
GUATEMALA	0.007
SALVADOR	?
HONDURAS	0.117
COSTA RICA	0.053
NICARAGUA	0.001
PANAMA	0.079

AIDS CASES 1989-90 REPORTED IN CARIBBEAN



SOURCE:WHO AIDS/HIV REPORT

AIDS CASES 1989-90

REPORTED BY CARIBBEAN COUNTRIES

COUNTRY	POPULATION	89	90
TRINIDAD /TOBAGO	1200000	336	557
ANTIGUA	81000	3	3
BAHAMAS	236000	236	437
BARBADOS	253900	67	112
SAINT VINCENT	117000..	14	22
CAYMANS	24900	4	5
CUBA	10,000000	43	63
DOMINICA	765000	6	10
DOM.REPUBLIC	6800000	619	1200
GRENADA	103400	10	14
GUADELOUPE	86	175
HAITI	5510917	1661	2331
JAMAICA	2355400	72	140
SAINT LUCIA	142400	11	16

SOURCE:WHO AIDS/HIV REPORT

AIDS CASES /1000 POPULATION

REPORTS FROM CARIBBEAN 90

COUNTRY	CASE/1000 POP
ANTIGUA	0.037
BAHAMAS	1.85
BARBADOS	0.441
CUBA	0.006
DOMINICA	0.131
DOMINICAN REPUBLIC	0.177
GRENADA	0.135
HAITI	0.423
JAMAICA	0.059
ST.LUCIA	0.112
ST.VINCENT	0.188
TRINIDAD TOBAGO	0.464

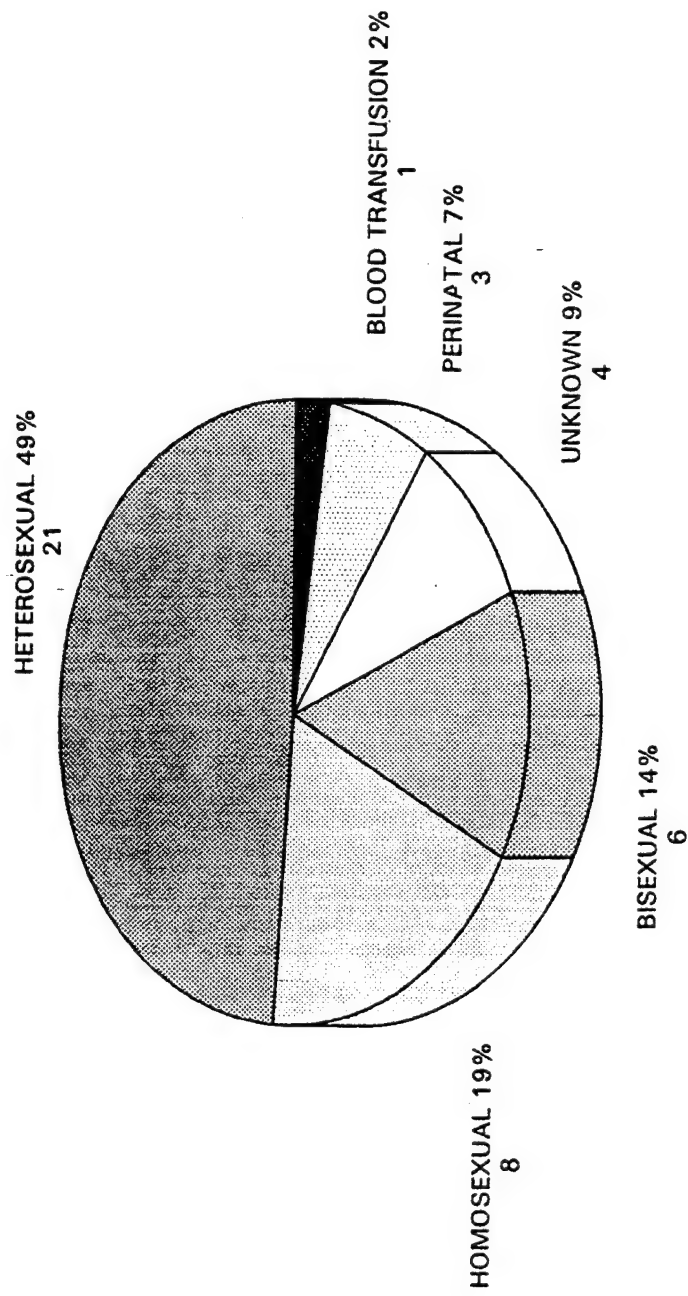
SOURCE WHO REPORT 90

AIDS/HIV Data System

- **Nurse venereologist**
- **voluntary anonymous testing?**
- **elisa screen/ western blot confirmatory**
- **std clinic, contacts, brothel, prison**
- **aids coordinator**
- **central statistic office**

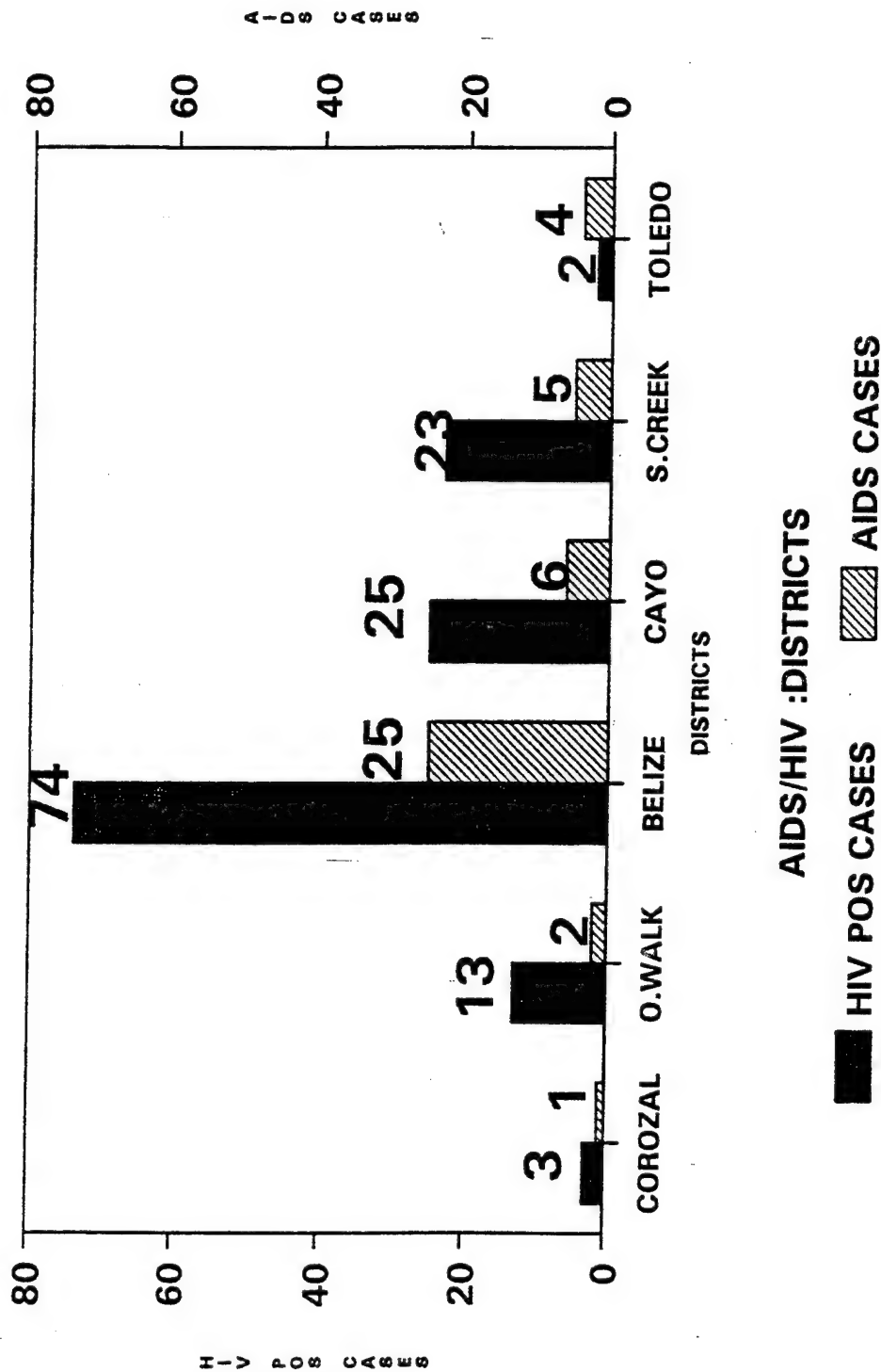
AIDS IN BELIZE

MODE OF TRANSMISSION



SOURCE: MOH BELIZE 91

HIV/AIDS REPORTED IN BELIZE 87-92 BY DISTRICT



SOURCE:MOH BELIZE 92

AIDS CASES DISTRIBUTION **BY AGE GROUP & SEX**

AGE GROUP	MALE	FEMALE	TOTAL
0-14	2	0	2
15-19	1	0	1
20-29	1	5	16
30-39	9	6	15
40-49	2	0	2
50-59	3	0	3
60 +	3	0	3
TOTAL	31	11	42

SOURCE: MOH BELIZE

HIV POS DISTRIBUTION

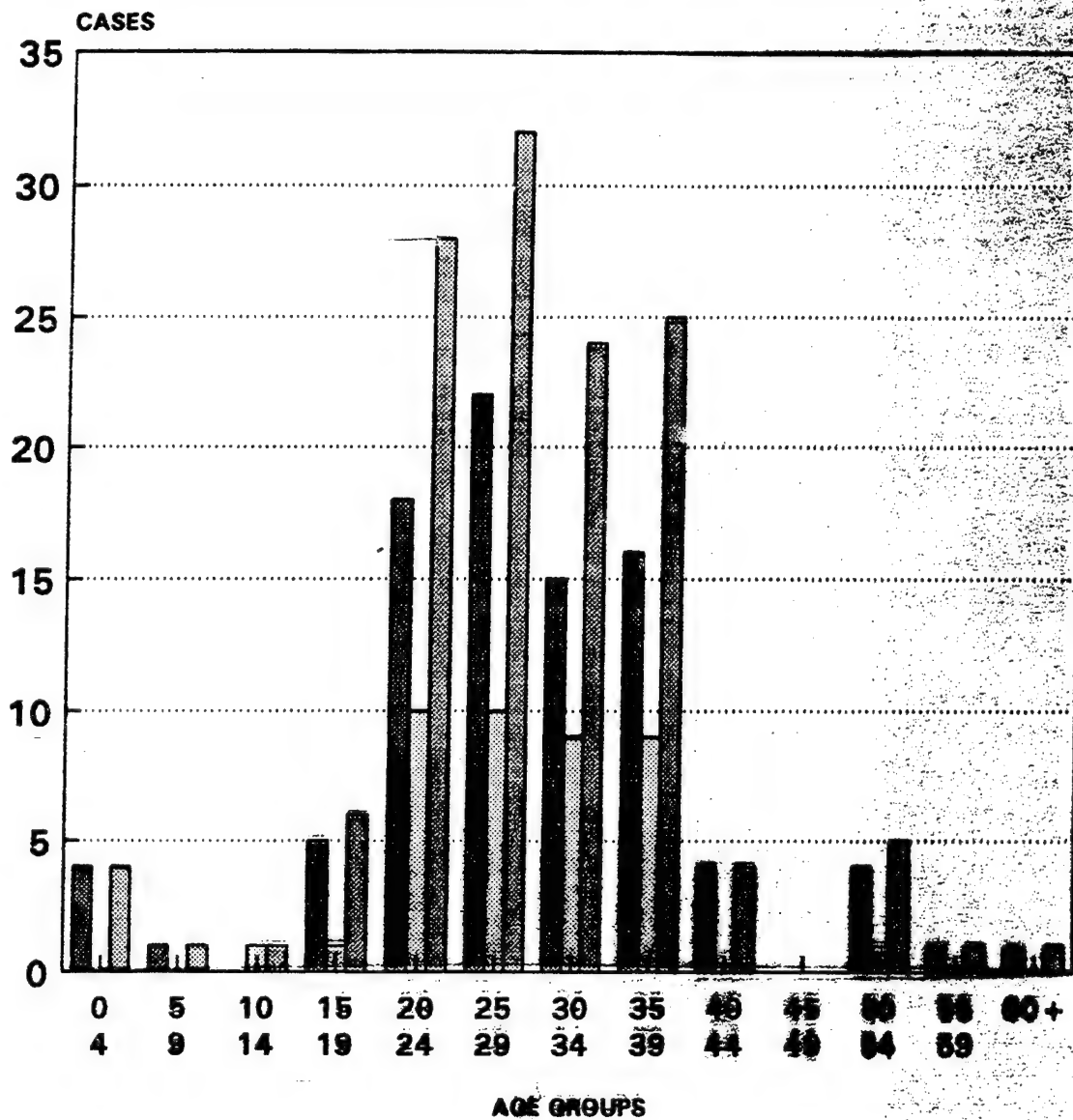
BY AGE GROUP & SEX

AGE GROUP	MALE	FEMALE	TOTAL
0-14	5	1	6
15-19	5	1	6
20-29	40	20	60
30-39	31	18	49
40-49	4	0	4
50-59	5	1	6
60+	1	0	1
total	91	41	132

TOTAL 91 41 132

SOURCE: MOH BELIZE

HIV POSITIVE DISTRIBUTION BY AGE GROUP AND SEX



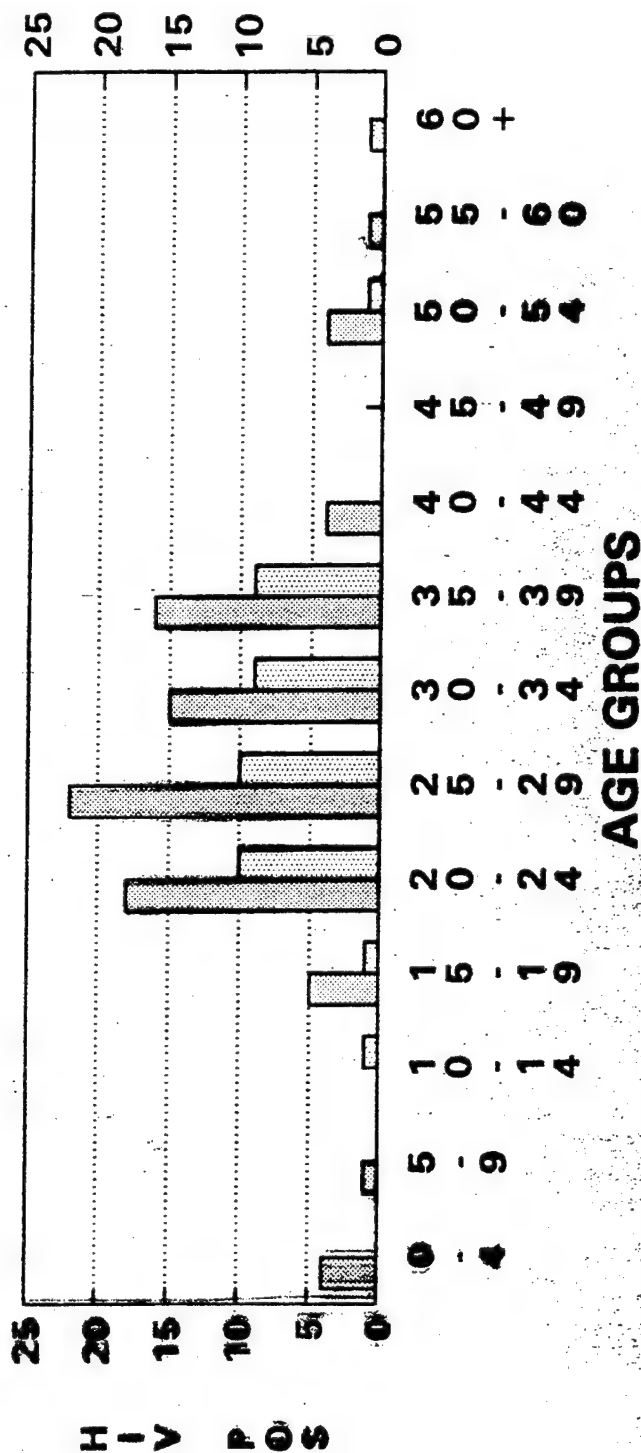
MALES

FEMALES

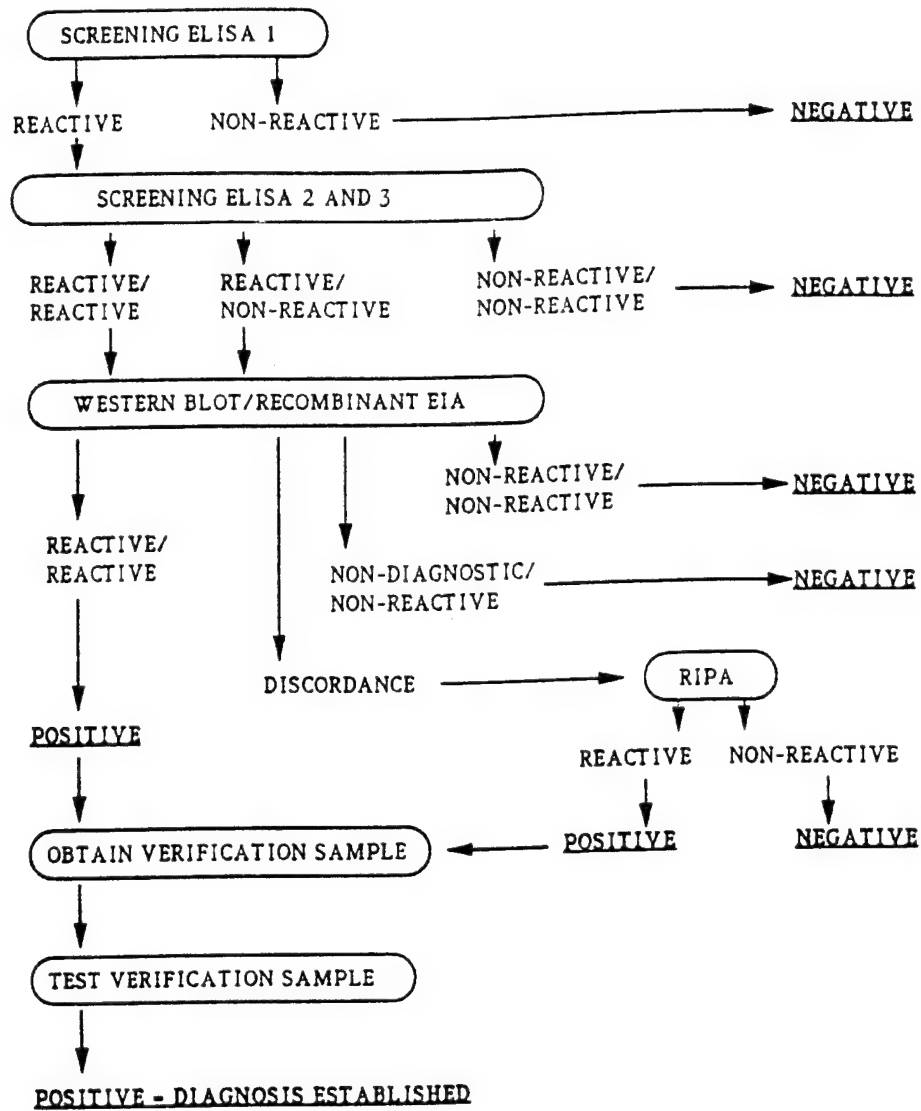
TOTAL

SOURCE: MOH BELIZE

HIV POS DISTRIBUTION BY AGE GROUP & SEX



SOURCE: MOH BELIZE



BELIZE NATIONAL AIDS PROGRAMME

HIV/AIDS REPORT

CONFIDENTIAL

First report to NAP for this patient? No / Yes. If previously reported, ID No.: _____

Surname: _____ First Name: _____ Middle Name: _____

Nickname: _____ Sex: M/F Occupation: _____

D.O.B.: _____ Age: _____ yrs/wks.(if infant) Marital Status: _____

Home address: _____

Town: _____ District: _____

If child, please enter MOTHER's name and address below

If adult, please enter Next of Kin or, if none available, any alternative address, work address or other identifying information below.

NOK/Alt.addr.

Surname: _____ First Name: _____

Address: _____

Town: _____ District: _____ Relat.to patient: _____

Other identifying information: _____

ELISA (1): submitted on: _____ Lab.: _____ Result: pos/ind/neg/pending

Suppl.test 1: Type: _____ Result: pos/ind/neg/pending

Suppl.test 2: Type: _____ Result: pos/ind/neg/pending

ELISA (2): submitted on: _____ Lab.: _____ Result: pos/ind/neg/pending

Suppl.test 1: Type: _____ Result: pos/ind/neg/pending

Suppl.test 2: Type: _____ Result: pos/ind/neg/pending

REASON TESTED/REPORTED: 'routine' / survey / risk / clinical suspicion / other _____

FOR CHILD ONLY: HIV status of MOTHER: Neg / pos / results awaited / not tested

MOTHER in high risk categ.: No/Yes/NK FATHER high risk categ.: No/Yes/NK

Haemophil.: No/Yes/NK Transf.blood/prod.: No/Yes/NK Hist.sexual abuse: No/Yes/NK

Other,specify: _____ Nationality: _____

FOR ADULT: Nationality: _____ Travel last 10 yrs: No/ Yes/ not known

If travelled:

Country(1): _____ Yr.travel: _____ Resident/visit/seasonal work

Country(2): _____ Yr.travel: _____ Resident/visit/seasonal work

(Please list information on any additional travel on a separate sheet)

Sexual practice: heterosexual/ homosexual/ bisexual/ not known

CONTINUED OVERLEAF

FOR ADULT: Known Risk behaviour in last 10 years:

Known heterosex.cont.with HIV pos: No/Yes/NK Known homosex.cont.with HIV pos: No/Yes/NK
Sex.cont. with FEMALE prostitute: No/Yes/NK Sex.cont. with MALE prostitute: No/Yes/NK
Haemophiliac: No/Yes/NK Transfusion blood or bl.product: No/Yes/NK
History of STD : _____ History of Genital Ulcers: No/Yes/NK
Intravenous drug abuse: No/Yes/NK Other, specify: _____

CURRENT STATUS OF PATIENT: HIV pos(no sympt.)/ HIV pos(some sympt.)/ AIDS/ AIDS death

CURRENT MEDICAL CARE: Attending Physician: _____

Hosp./Clinic: _____ Ward/Dept.: _____ Docket/I.D.No.: _____

CLINICAL INFORMATION: If HIV related symptoms present, clinically AIDS case or AIDS death,

Date onset symptoms: _____ Date diag.as AIDS: _____ Date death: _____

In the CURRENT ILLNESS EPISODE, does the patient have:

Fever (for >1 month): No/Yes/NK Pulm.Tuberculosis: No/Yes/NK
Cough (for >1 month): No/Yes/NK Generalised dermatitis: No/Yes/NK
Shortness of breath: No/Yes/NK Shingles: No/Yes/NK
Weight loss (>10% body wt.): No/Yes/NK Candidiasis: No/Yes/NK
Diarrhoea (for >1 month): No/Yes/NK Kaposi's Sarcoma: No/Yes/NK
Lymphadenopathy (>2 sites): No/Yes/NK CNS involvement: No/Yes/NK

If CNS involvement, specify: _____

Other STD, specify: _____

Other, specify: _____

CONTACTS: Surname First name Relationship Address

(Please list additional contacts on a separate sheet)

Date: _____ Reported by: _____

Proposed AIDS/HIV surveillance instrument

ID number-----

SEX -----

occupation -----

DOB(month, year) -----

ADDRESS(town, district)-----

MARITAL STATUS-----

TEST RESULTS-----

OTHER PARAMETERS

BLOOD donor?YES or NO

ANY BLOOD TRTANSFUSION-----

AGE LAST CHILD-----

HISTORY STD'S-----

HISTORY OF tatooing or blood letting-----

SEXUAL PRACTICES-----

DRUG ABUSE? YES OR NO ,IF YES STATE NATURE OF ABUSE

CLINICAL STATUS OF PERSON-----

ATTENDING PHYSICIAN -----

TREATMENT MODALITY -----

Pesticide avoidance behavior in Anopheles albimanus, a malaria vector in Central and
South America

by

Theeraphap Chareonviriyaphap

Dissertation submitted to the Faculty of the Department of Preventive Medicine and
Biometrics Graduate Program of the Uniformed Services University of the Health Sciences
in partial fulfillment of the requirements for the degree of Doctor of Philosophy 1995

COMMUNITY AND ECOSYSTEM ECOLOGY

Environmental and Regional Determinants of *Anopheles*
(Diptera: Culicidae) Larval Distribution in Belize,
Central America

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S. MANGUIN,¹ R. KRIEG,³ J. POLANCO,⁴ AND L. LECTERS¹

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ABSTRACT Surveys of *Anopheles* larval habitats in northern Belize were carried out during September 1990 and April 1991. At each site, larvae were collected and the physical and chemical characteristics of water and species composition of aquatic vegetation were measured or estimated. Data on presence or absence of four species, *Anopheles albimanus* Wiedemann, *A. crucians* Wiedemann, *A. pseudopunctipennis* Theobald, and *A. argyritarsis* Robineau-Desvoidy, were used for analysis of associations with environmental factors, habitat types, and regions. Using significantly contributing environmental variables, discriminant functions (DF) were constructed for the *Anopheles* species, except for *A. argyritarsis* whose distribution could be predicted solely by altitude. The stability of DFs was checked by cross-validation runs. The DF for *A. pseudopunctipennis* was 93% accurate in predicting positive habitats. Predictions based on DFs for *A. albimanus* and *A. crucians* were 74 and 80% accurate, respectively. Of the four *Anopheles* species present in the study area, *A. albimanus* was the most common. Together with *A. crucians*, it occurred mostly on the coastal plain, and both species were present in both wet and dry seasons. *Anopheles albimanus* was positively associated with cyanobacterial mats and submersed-periphyton habitat types and negatively associated with the filamentous algae habitat type. *A. crucians* was positively associated with *Eleocharis*-periphyton habitat type. *A. pseudopunctipennis* and *A. argyritarsis* were common only during the dry season and their distribution was limited to the Karst and Mountain Pine Ridge regions. Both species were positively associated with the filamentous algae habitat type, and *A. argyritarsis* was also positively associated with the rock pools habitat type. Physical factors (e.g., water depth, water temperature, and oxygen content) were usually marginally correlated with larval occurrence. Dominant plant growth forms, such as filamentous algae, cyanobacterial mats, and submersed macrophytes showed the closest association with the larvae of particular *Anopheles* species. Our results demonstrated the controlling influence of dominant aquatic vegetation on larval presence.

KEY WORDS larval habitats, aquatic vegetation, *Anopheles* spp.

GEOMORPHOLOGY affects the hydrology of a region; i.e., distribution and seasonal dynamics of lakes, rivers, streams, and pools. Water quality in these different water bodies is influenced by rock and soil chemistry, vegetation of the surrounding landscape, and human activities. Both hydrology and water chemistry determine the type of aquatic vegetation present in lakes, pools,

and streams. Shallow, quiet water with aquatic vegetation seems optimal for oviposition and larval development of most mosquito species. Descriptions of requirements of individual species for specific characteristics of larval habitats have generally been rather vague. A few attempts to describe the relationships between larvae and different environmental factors can be found in papers by Rioux et al. (1968), Hagstrum & Gunstream (1971), Hall (1972), Vrtiska & Pappas (1984), Gabinaud (1987), Orr & Resh (1989), Savage et al. (1990), and Rejmankova et al. (1991).

Obviously, if we can point out individual environmental factors related to the presence of larvae, then groups of individual factors are probably characteristic of specific larval habitats, which, in turn, might be related to distinct geo-

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graphic regions. Once the connections are made between the fine scale of individual larval habitats and a coarse scale of their regional distribution, our understanding of mosquito larval ecology, specifically with regard to malaria transmission, will be greatly improved.

The country of Belize, located south of the Yucatan Peninsula on the Atlantic coast of Central America (Fig. 1), provides a great variety of ecological settings as foci of malaria transmission. Komp (1941) first reported the occurrence of *Anopheles darlingi* Root in Belize. This finding was verified by Kumm & Ram (1941), who also documented the occurrence of malaria-infected specimens of *A. darlingi* and *A. vestitipennis* Dyar & Knab. Additionally, Kumm & Ram (1941) reported the presence of seven other species of *Anopheles*; i.e., *A. albimanus* Wiedemann, *A. pseudopunctipennis* Theobald, *A. punctimacula* Dyar & Knab, *A. apicimacula* Dyar & Knab, *A. eiseni* Coquillett, *A. argyritarsis* Robineau-Desvoidy, and *A. crucians* Wiedemann. Bertram (1971) reported collecting all of these species, except *A. darlingi*, in Belize. Bertram's work, which emphasized the ecology of adult mosquitoes, is practically the only source of information on the spatial and seasonal distribution of anophelines in Belize.

Not only in Belize but throughout Central America, larval ecology of malaria vectors has been the subject of infrequent and sporadic studies. Review papers by Rozeboom (1941), Watson & Hewitt (1941), and Bates (1949) described the seasonal and spatial distribution of *A. albimanus* and *A. pseudopunctipennis*. Breeland (1972) presented specific information on the seasonal and spatial distribution of these vectors along the Pacific coast of El Salvador. Bailey et al. (1981) studied the distribution of *A. albimanus* larvae in estuarine habitats of El Salvador. The relationships of *A. albimanus* and *A. pseudopunctipennis* larvae to dominant aquatic plants and environmental factors in southern Chiapas, Mexico, have been reported by Savage et al. (1990) and Rejmankova et al. (1991). A hierarchical method for classifying larval habitats into habitat types was subsequently suggested by Rejmankova et al. (1992).

In addition to *A. albimanus* and *A. pseudopunctipennis*, several other *Anopheles* occur in Central America. Recently, *A. vestitipennis*, previously considered to be a relatively unimportant malaria vector, was found to transmit malaria in areas within Mexico and Guatemala (Loyola et al. 1991, Padilla et al. 1992). Roberts et al. (1993) found this species to be of potential importance as a vector of malaria in Belize. These recent findings are indicators of our poor understanding of vectorial roles of *Anopheles* in much of Central America. Malaria rates in Belize are increasing, so the issues of species distributions and

vectorial roles are increasingly important to the health and welfare of the Belizean population.

An array of vegetation types exists in Belize. Most of the primary tropical deciduous forests have been disturbed by intensive logging for mahogany and logwood and traditional slash-and-burn agriculture. Extensive areas on the coastal plain are covered with seasonally inundated savanna, lowland pine forest, and freshwater swamp forest. Mangrove swamps are common along the coast and extend inland wherever brackish water occurs. Sugarcane, grown mostly in northern Belize, is a prime agricultural crop. Citrus-growing is becoming more important, with large areas of forest in the Cayo and Stann Creek districts currently being cleared for citrus cultivation.

In September 1990, we initiated a surveillance program to obtain population-based data on the malaria vectors in Belize. The quantity of environmental data compiled was greater than normally collected in field surveys. This allowed the larval-environmental associations to be studied from different levels of detail, ranging from the individual habitat to a regional level. The most detailed analysis was performed at the individual habitat level, using environmental variables that might affect oviposition as well as larval distribution, density, development, and survival. A second approach was based on a more holistic view of larval habitats. Using this approach, habitats were described according to their dominant vegetation, classified into habitat types, then examined for association between habitat types and the presence or absence of *Anopheles* species. The third approach to data analysis involved assessment of associations at the regional level.

Program objectives were to document which vector species were present in northern Belize, to define the habitat ranges of these species, and to determine whether their presence or absence could be predicted by environmental factors, habitat types, or regional characteristics. Reported herein are the results of habitat analysis and regional distribution of *A. albimanus*, *A. pseudopunctipennis*, *A. crucians*, and *A. argyritarsis*.

Materials and Methods

Study Area. With an area of 23,000 km² and a population of ≈180,000, Belize is a country with the lowest population density in Central America. Lowlands of Belize are characterized by a variety of wetlands, freshwater and brackish, seasonal and permanent. Montane and foothill regions include many streams and rivers. The hydrological and vegetational diversity results in a wide variety of mosquito larval habitats.

The amount of rainfall increases from ≈1,300 mm annually in the north to 2,400 mm around Belize City. The normal dry season is from January through April and is shorter and less severe

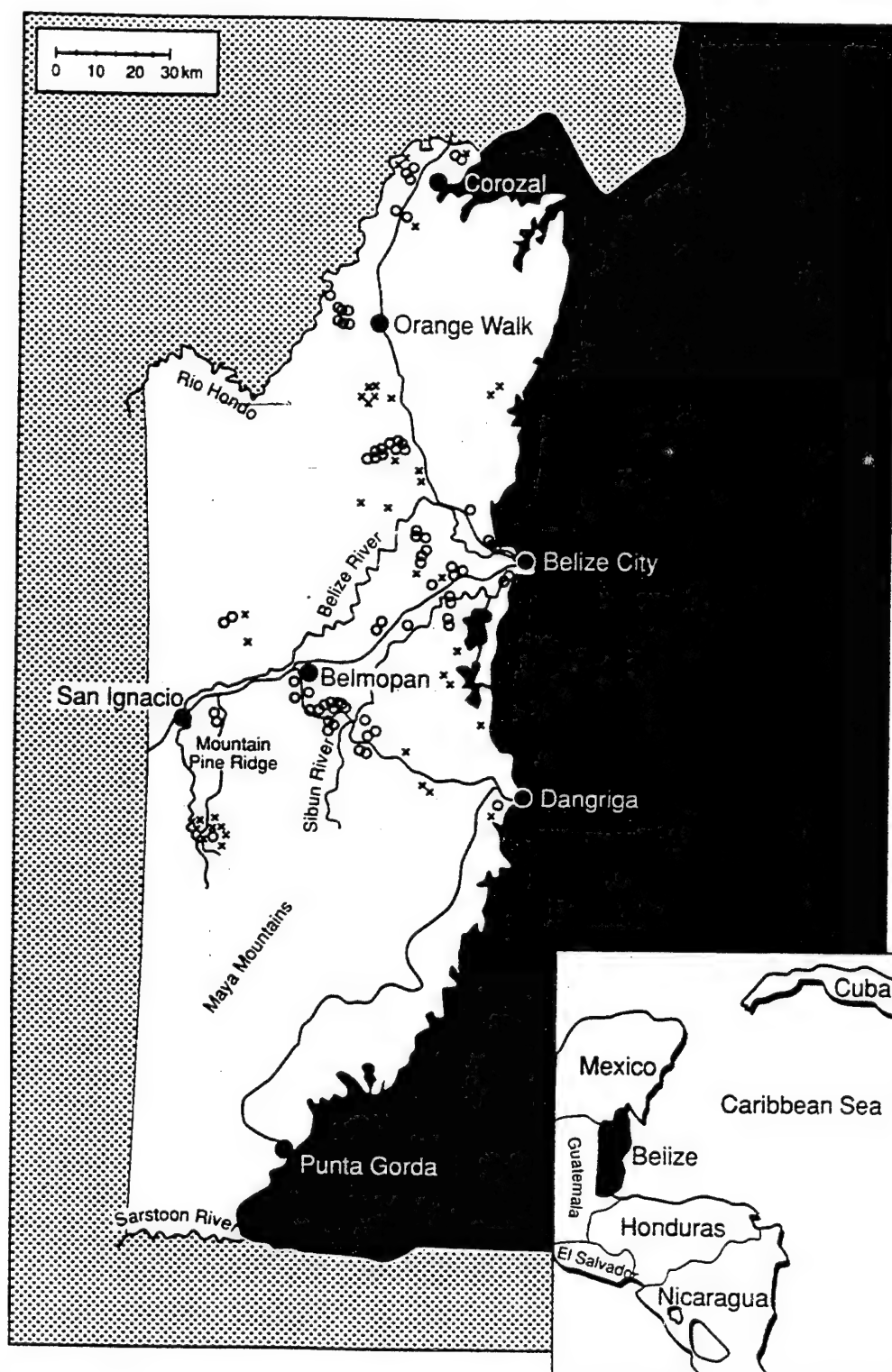


Fig. 1. Map of Belize with location of sampling sites in the wet (September 1990) and dry (April 1991) seasons. Circles indicate sites visited during both seasons. Crosses indicate sites added in the dry season.

than at comparable latitudes on the Pacific coast of Central America.

Our survey sites were distributed in the northern part of Belize from Dangriga north, covering Corozal, Belize, Orange Walk, and parts of Cayo and Stann Creek districts (Fig. 1). This northern area includes three distinct physiographic regions: flat coastal and inland plain (CP), karst and foothills (KARST), and Mountain Pine Ridge (MPR), which all differ in their topography, geology, hydrology, soils and, consequently, vegetation cover (Hartshorn et al. 1984). The terms "regional" and "region" are used in this article for physiographic regions of northern Belize on a scale of 10^2 – 10^3 km².

The MPR region includes fast-flowing rivers and streams with nutrient-poor waters of very low mineral content. No extensive wetlands occur in this region; therefore, mosquito habitats exist in the form of river pools with filamentous algae and occasional graminoids. In the KARST region, larval habitats are also mostly associated with rivers. These rivers are slower than in the MPR and their waters are richer in minerals, specifically calcium. Pasture ponds and small lagoons with different types of aquatic vegetation are also present in this region. The CP includes both fresh and brackish waters and provides very diverse and often extensive habitats ranging from almost monospecific marshes dominated by a sedge, *Eleocharis interstincta* (nomenclature for vascular plants follows Standley & Steyermark 1946–1977), to species-rich ponds and lagoons.

Larval Sampling. Surveys of a wide range of mosquito larval habitats were conducted in the northern part of Belize in both the wet (September 1990) and dry (April 1991) seasons. A mosquito larval habitat is defined as a body of water with uniform vegetation and a specific water chemistry (Rejmankova et al. 1992). In the wet season, larval habitats were sampled at 75 different sites (see Fig. 1). In the dry season, the 75 sites previously surveyed in the wet season were visited and some new sites were added because many of the wet-season locations were dry. The total number of sites with water in the dry season including both the old and added sites was 73.

The following data were recorded for each habitat: total percentage of emergent, floating, or submersed vegetation, algal mats, and detritus; percentage of cover of individual plant species; amount of phytoplankton (measured fluorometrically as chlorophyll *a* concentration); water conductivity; pH; and dissolved oxygen. Water analyses were conducted for total suspended solids, particulate organic matter, nitrate and ammonia nitrogen (NO₃, NH₄), orthophosphate phosphorus (PO₄), and major cations (Na, K, Ca, Mg) using standard limnological methods (APHA 1985). Thirty dips for mosquito larvae were taken from each habitat. Although a greater number of dips was not practically feasible, we already

knew from earlier work that 30 dips provided a rough estimate of population density (Savage et al. 1990; Rejmankova et al. 1991). To process the samples, larvae and pupae were transported to the laboratory in Belize City and reared to obtain adults with associated immature exuviae for identification, study, and future reference. Some fourth instars were also preserved from most collections.

Larval Occurrence and Environmental Factors. Data on the occurrences of larvae of different *Anopheles* species were related to environmental factors. Because of large variations in larval density, most analyses were conducted using information on the presence-absence of individual species. The environmental variables were subjected to either log transformation (conductivity) or the angular transformation (all plant variables were expressed as percentage values) before further analysis. The two-tailed *t* test was used to compare the group means of environmental variables for sites with or without larvae.

Discriminant Analysis. Relationships between the presence-absence of each *Anopheles* species in the dry season data set and the selected environmental variables were further explored using discriminant analysis (Tabachnick & Fidell 1989). Our goal was to select a reduced set of variables for predicting the distribution of each species. The discriminant functions were first calculated using all the environmental variables identified by *t* test as having significantly different group means for sites with and without larvae. Subsequently, the variables that did not contribute significantly to the respective discriminant functions were deleted. The final number of variables used was four for *A. albimanus* and *A. crucians* and three for *A. pseudopunctipennis*. We did not calculate the discriminant function for *A. argyritarsis*, whose distribution could be predicted solely by altitude.

To assess the predictive power of the respective discriminant functions, five randomly selected subsets of data were used to calculate the functions that were subsequently applied to independent data subsets (cross-validation technique; see Tabachnick & Fidell 1989).

Habitat Types. Because of substantial habitat diversity, the individual habitats, defined by dominant plant species, were categorized into higher units, subsequently referred to as habitat types (Rejmankova et al. 1992). Cluster analysis (Orloci 1978) based on the absolute distance dissimilarity after the angular transformation of the environmental variables (plant cover) was used for delineation of nine habitat types based on the wet-season data. During dry-season sampling, a site was ascribed to a habitat type before sampling for larvae was done. Three additional distinctive habitat types were sampled in the dry season: rock pools without filamentous algae, detritus, and planktonic algae. Based on the aver-

Table 1. Average specific conductivity \pm SD for sampling sites in the four regions in the wet and dry seasons

Season	Mountain pine ridge	Karst	Coastal plain	
			Fresh	Brackish
Wet	14	144 \pm 129	91 \pm 71	2,186 \pm 820
Dry	42 \pm 12	198 \pm 216	183 \pm 97	1,826 \pm 891

age number of larvae per dip, the habitat types were ranked as high (>1), medium (0.1–1), and low (<0.1).

Larval Distribution Within Defined Habitat Types and Geographic Regions. G tests of independence (Zar 1984) were calculated to determine the associations between the presence-absence of each vector species and habitat types and regions, respectively.

Results

Dry-season sampling revealed that 66% of wet-season habitats were dry during the dry season, 7% were significantly smaller, and 27% were relatively unchanged. Water conductivity was significantly higher in the dry season in habitats in both MPR and CP (fresh), whereas it did not differ much in KARST and CP (brackish) (Table 1). Plant diversity was much higher in the wet season than in the dry season (see list of plant species in Appendix 1), mainly because species-rich edges of ponds and lagoons that were flooded during the wet season dried up and ceased being larval habitats during the dry season.

Larval Occurrence and Environmental Factors. Physical factors (e.g., water depth, water temperature, oxygen content) were usually marginally correlated with larval occurrence. Dominant plant growth forms such as filamentous algae, cyanobacterial mats, and submersed macrophytes showed the closest association with the larvae of particular *Anopheles* species.

Discriminant Analysis. Using the environmental variables with significantly different group means for sites with larvae present versus absent (Tables 2 and 3), we calculated discriminant functions for the dry season for all the *Anopheles* species (Fig. 2 a–c), except for *A. argyritarsis*.

For *A. albimanus*, 10 environmental variables were significantly different for dry-season sites with and without larvae (Table 2). Of these variables, only cover percentage of submersed plants, cover percentage of cyanobacterial mats, altitude, and temperature contributed significantly to the discriminant function by 44, 30, 14, and 12%, respectively. The discriminant function for the whole data set correctly predicted the presence of larvae in 74% of all sites and correctly predicted the absence of larvae in 91% of the sites (Fig. 2a; Table 4). Five randomly selected subsets of data were then used to con-

struct the discriminant functions. When these functions were tested on the remaining independent subsets of data, the correctly predicted percentage of sites with larvae varied from 45 to 89%. Additionally, 81–95% of sites without larvae were correctly classified (Table 4). Cover percentage of periphyton, detritus, and emergent plants and habitat area were used as variables for constructing discriminant functions for *A. crucians* (Fig. 2b; Table 5) contributing by 44, 24, 22, and 10%, respectively, to the predictive power of the DF. Using the entire data set, the function correctly classified 80% of sites for the presence of larvae and 94% for the absence of larvae. Using five randomly selected subsets, correct predictions varied from 33 to 100% and from 84 to 91% for presence and absence of larvae, respectively. Cover percentage of filamentous algae, altitude, and water depth were used to construct the discriminant function for *A. pseudopunctipennis* (Fig. 2c; Table 6) contributing 81, 11, and 8%, respectively, to the predictive power of the DF. With the entire data set, the function correctly classified 93% of the sites for presence of larvae and 93% for absence of larvae. For the five randomly selected subsets, correct predictions ranged from 78 to 100% for positive sites and from 87 to 100% for negative sites.

Habitat Types. During the wet and dry season collections, nine and 12 major habitat-types were distinguished respectively, as defined by a dominant plant species, genera or life form (Fig. 3; Table 7). Of these twelve habitat types, five represented emergent macrophytes (including mangroves), two belonged to floating hydrophytes, and three were characterized by submersed hydrophytes. Three remaining habitat types (rock pools with no filamentous algae, detritus, and planktonic algae) did not contain any macrophytic vegetation. The detailed description of habitat types is given in Appendix 2. As shown in Table 7, habitat types cyanobacterial mats, submersed macrophytes-periphyton, *Nymphaea-Limnanthemum*, and mangroves were relatively stable with three of five, two of five, three of seven, and two of five sites staying the same in both the wet and dry seasons, respectively. Most sites sampled during the wet season that belonged to graminoids and *Eleocharis interstincta*-periphyton habitat types and all sites of *Typha-Cladium* habitat type were dry during the dry season. Three sites of wet-season graminoids habitat type, two sites of cyanobacterial mats habitat type, and one site each of *Eleocharis interstincta*-periphyton and *Nymphaea-Limnanthemum* habitat types developed into different habitat types during the transition from wet to dry seasons.

Larval Distribution Among Habitat Types. The tendency of water bodies to contain the same habitat type in both seasons (16 of 25) and, consequently, to support larvae of the same spe-

Table 2. Comparison of significantly different group means (\pm SD) of environmental variables measured in the dry season (two-tailed *t* test)

Environmental variable	Larvae		P < *
	Present	Absent	
<i>Anopheles albimanus</i>			
No. sites	27	46	—
% Cyanobacterial mats	20.4 (31.5)	1.8 (10.3)	0.0001*
% Submersed	29.0 (38.1)	2.1 (11.7)	0.0001*
% Filamentous algae	3.3 (17.3)	17.4 (28.0)	0.005*
Habitat area, m ²	25.6 (41.3)	7.5 (11.2)	0.006
Water body area, m ²	1,848.8 (2,600.0)	644.8 (1,200.0)	0.009
% Periphyton	5.8 (16.1)	0.6 (1.5)	0.01*
Altitude, m	35.3 (56.9)	118.3 (171.8)	0.02
Conductivity μ mhos/m	1,086.5 (1,087.0)	738.9 (1,610.6)	0.03+
Temperature, °C	32.8 (2.1)	31.6 (1.9)	0.03
Oxygen, ppm	8.6 (3.7)	7.1 (2.6)	0.058
<i>Anopheles crucians</i>			
No. sites	10	63	—
% Periphyton	12.9 (25.3)	0.9 (2.2)	0.0001*
% Emerged	28.2 (28.9)	11.4 (23.0)	0.01*
% Detritus	12.0 (28.2)	1.9 (9.7)	0.02*
Habitat area, m ²	30.0 (60.2)	11.6 (18.0)	0.05
<i>Anopheles pseudopunctipennis</i>			
No. sites	14	59	—
% Filamentous algae	48.9 (31.1)	3.5 (13.5)	0.0001*
Conductivity, μ mhos/m	102.0 (94.6)	1,049.1 (1,549.5)	0.0002+
Water depth, cm	8.0 (9.45)	24.0 (15.5)	0.0004
Altitude, m	192.1 (178.0)	62.7 (126.4)	0.002
% Emerged	0.07 (0.26)	17.0 (26.1)	0.004*
Temperature, °C	30.9 (2.5)	32.3 (1.9)	0.02
Water body area, m ²	1.8 (2.6)	1,348.0 (2,054.0)	0.02
% Submersed	0.0 (0.0)	14.9 (30.4)	0.03*
Habitat area, m ²	1.4 (1.5)	17.1 (30.1)	0.05
<i>Anopheles argyritarsis</i>			
No. sites	9	64	—
Altitude, m	453.0 (40.0)	36.0 (47.5)	0.0001
Conductivity, μ mhos/m	42.2 (12.7)	983.5 (1,504.2)	0.0001+
Temperature, °C	33.9 (1.6)	31.8 (2.0)	0.005
% Emerged	0.11 (0.33)	15.6 (25.5)	0.03*
Water body area, m ²	0.8 (0.8)	1,243.3 (2,004.6)	0.058

* *, after angular transformation; +, after log transformation.

Table 3. Comparison of significantly different group means (\pm SD) of environmental variables measured in the wet season (two-tailed t test)

Season (two-tailed t test)			
Environmental variable ^a	Larvae		P < ^b
	Present	Absent	
<i>Anopheles albimanus</i>			
No. sites	18	57	—
% Cyanobacterial mats	24.4 (24.5)	3.51 (7.26)	0.0001*
POM, ppm	53.26 (94.84)	3.55 (4.19)	0.0001
Ca ⁺⁺ , ppm	168.41 (206.26)	52.67 (80.75)	0.0009
TSS, ppm	69.92 (121.27)	9.77 (11.16)	0.0004
Mg ⁺⁺ , ppm	54.65 (67.25)	16.91 (31.85)	0.002
% Detritus	8.16 (16.05)	1.28 (4.34)	0.003*
pH	7.57 (0.63)	7.10 (0.80)	0.03
<i>Anopheles crucians</i>			
No. sites	9	66	—
pH	6.63 (0.76)	7.30 (0.76)	0.02

^a POM, particulate organic matter; TSS, total suspended solids.

^b *, after angular transformation; +, after log transformation.

cies, was significant (G test; $P < 0.025$). Larval density was much higher in the dry season than in the wet season (Fig. 3). All four *Anopheles* species were present in the dry season, whereas only *A. albimanus* and *A. crucians* were found in the wet season. In the dry season, cyanobacterial mats, filamentous algae, and submersed-periphyton represented high larval density habitat types (>1 larva per dip); *Eleocharis*-periphyton, broadleaved, rock pools, detritus, and planktonic algae belonged to medium-density habitat types (0.1–1 larva per dip); and the rest were low-density habitat types (<0.1 larva per dip). In the wet season, high densities of larvae were found in cyanobacterial mats and filamentous algae habitat types, the graminoids habitat type produced medium numbers of larvae, and the remaining habitat types produced very few larvae. Because of a large variability in larval counts and a low number of replicates, we did not find statistically significant differences in larval density between habitat types (Scheffe multiple comparison test), except for a wet-season difference between cyanobacterial mats and all remaining habitat-types.

The results of the G test of independence between habitat types and *Anopheles* species (Fig. 4) show a highly significant positive association between *A. albimanus* and the cyanobacterial mats and submersed-periphyton habitat types, and a highly significant negative association between *A. albimanus* and filamentous algae habitat type. *A. crucians* was positively associated with the *Eleocharis*-periphyton habitat type and slightly negatively associated with the filamentous algae habitat type. *A. pseudopunctipennis*

and *A. argyritarsis* were positively associated with the filamentous algae habitat type, and *A. argyritarsis* was positively associated with the rock pools (no algae) habitat type.

Regional Distribution. Fig. 5 summarizes the data on distribution of *Anopheles* species among the different regions of the study area. *A. argyritarsis* was found only in rock pools of MPR. The rock pools are characterized by low water conductivity with very low content of minerals. *A. pseudopunctipennis* occurred in both MPR and KARST, always in river pools with filamentous algae. Water in the KARST region has a higher content of minerals, specifically calcium (>20 ppm). *A. crucians* was found mainly in habitats associated with CP (fresh) (water conductivity comparable to KARST), even though it was occasionally present in KARST and CP (brackish) as well. The highest larval densities of *A. albimanus* were found in habitats of CP (brackish), but this species was quite common in KARST and CP (fresh) as well. Statistical significance of these associations is expressed in Fig. 6.

Discussion

Discriminant Functions. The discriminant functions for presence of *A. albimanus* and *A. pseudopunctipennis* using data from southern Chiapas, Mexico, were published by Savage et al. (1990). The authors used slightly different techniques to construct their DF and select the significant variables. Yet the final selection of important variables for *A. pseudopunctipennis* was the same as in this paper; i.e., filamentous algae, altitude, and water depth. Consequently,

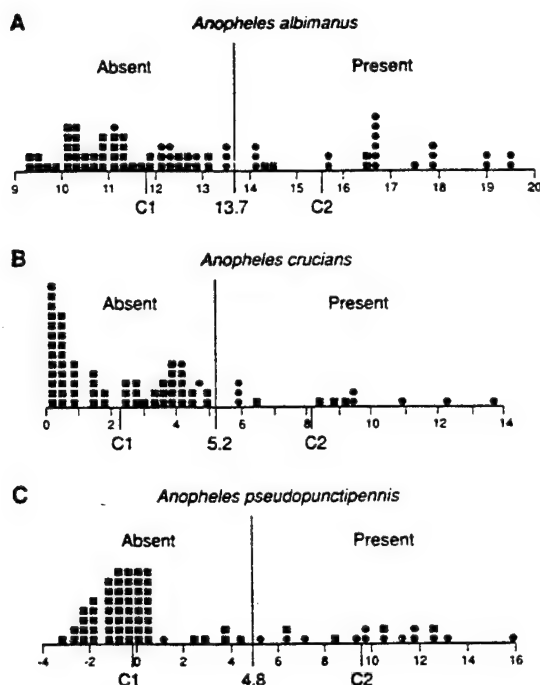


Fig. 2. (A) Discriminant function (Z , see Table 4) for *A. albimanus*. ■, Species absent; ○, species present. C1 and C2, Group centroids. (B) Discriminant function (Z , see Table 5) for *A. crucians*. ■, Species absent; ○, species present. C1 and C2, Group centroids. (C) Discriminant function (Z , see Table 6) for *A. pseudopunctipennis*. ■, Species absent; ○, species present. C1 and C2, Group centroids.

we are quite confident that the DF for *A. pseudopunctipennis* is broadly applicable to other northern areas of Central America. The environmental variables used, especially the cover percentage of filamentous algae that contributes $\approx 80\%$ to the predictive power of the DF, appear to exert a controlling influence on the distribution of this species.

Predictions based on the DF for *A. albimanus* were less accurate than those for *A. pseudopunc-*

tipennis. The DF for *A. albimanus* could not be compared with that of Savage et al. (1990) because their function included the cover of *Eichhornia*, a floating aquatic macrophyte, as one variable; *Eichhornia* was not found in Belize. The variables selected for DF were, in descending order of importance, submersed macrophytes, cyanobacterial mats, altitude, and water temperature. When the DF derived from the dry-season data was applied to the wet-season data set, it resulted in 72% of correctly predicted positive sites and 49% of correctly predicted sites with larvae absent. This is (at least for the positive sites) in the range of predictive values found for *A. albimanus*. The lower predictive value of *A. albimanus* DFs compared with DFs for *A. pseudopunctipennis* may be caused by the broader range of environmental conditions under which *A. albimanus* larvae occur. Variables associated with the presence of *A. albimanus* larvae in habitats in Belize were quite different from those in Mexico. In Mexico, the main variables were phytoplankton (unicellular green algae) in both seasons and *Eichhornia* in the dry season and Cyperaceae and phosphates in the wet season. None of these variables was linked with the distribution of *A. albimanus* larvae in Belize. Few habitats supported measurable quantities of phytoplankton in Belize, whereas many habitats were rich in phytoplankton in Mexico. This may be because waters in southern Chiapas contained generally 2–3 times higher concentrations of major nutrients (nitrogen, phosphorus) because of the volcanic origin of nutrient-rich soils in the area, abundant cattle manure, and extensive use of fertilizers. In the limestone regions of Belize, waters were poor in nitrogen and phosphorus but rich in calcium, both conditions being rather unfavorable for the growth of phytoplankton. On the other hand, extensive benthic cyanobacterial mats capable of nitrogen fixation, and submersed macrophytes overgrown with periphyton, were quite common in Belize, but they were not encountered in habitats in Mexico. Stands of several Cyperaceae species

Table 4. Cross-validation of discriminant functions for *A. albimanus* using five randomly selected data subsets

Subset no.	Derived from P/A ^a	Applied to P/A ^a	% Correctly predicted		Coefficients				Cut-off value
			As present	As absent	d_1	d_2	d_3	d_4	
1	18/19	9/27	89	81	0.526	-0.008	3.789	2.578	17.57
2	16/21	11/25	45	92	0.946	-0.008	4.624	6.208	32.61
3	9/28	18/18	72	94	0.263	-0.005	5.904	7.262	10.68
4	13/24	14/22	64	91	0.297	-0.005	3.994	3.947	11.08
5	11/26	16/20	69	95	-0.121	-0.003	7.007	3.400	-2.24
The whole data set			74	91	0.387	-0.006	4.428	4.306	13.68

General form of equation: $Z = d_1(T) + d_2(Alt) + d_3\arcsin(SB)^{1/2} + d_4\arcsin(BG)^{1/2}$, where T, temperature (°C); Alt, altitude (m); SB, submersed macrophytes (cover percentage after angular transformation); BG, cyanobacterial mats (cover percentage after angular transformation).

^a P, number of sites with species present; A, number of sites with species absent.

Table 5. Cross-validation of discriminant functions for *A. crucians* using five randomly selected data subsets

Subset no.	Derived from P/A ^a	Applied to P/A ^a	% Correctly predicted		Coefficients				Cut-off value
			As present	As absent	d ₁	d ₂	d ₃	d ₄	
1	6/31	4/32	50	84	0.028	5.409	24.154	8.004	5.80
2	6/31	4/32	50	91	0.043	3.663	15.128	18.039	6.92
3	6/31	4/32	75	91	0.006	4.846	11.347	4.852	3.74
4	5/32	5/31	100	91	0.014	3.111	5.527	7.613	2.93
5	4/33	6/30	33	93	0.002	4.207	13.738	21.631	6.24
The whole data set			80	94	0.034	4.785	13.429	9.712	5.20

General form of equation: $Z = d_1(HA) + d_2\arcsin(EM)^{1/2} + d_3\arcsin(PER)^{1/2} + d_4\arcsin(DET)^{1/2}$, where HA, habitat area (m²); EM, emergent macrophytes (cover percentage after angular transformation); PER, periphyton (cover percentage after angular transformation); DET, detritus (cover percentage after angular transformation).

^a A, number of sites with the species absent; P, number of sites with the species present.

were present in Belize, but they did not support comparably high densities of *A. albimanus* larvae as in Mexico.

The DF for *A. crucians* was about as accurate as the DF for *A. albimanus*. Similarities in predictive accuracy of DFs for the two species reflect the tolerance of both species to a wide variety of habitats.

The fourth *Anopheles* included in the analysis, *A. argyritarsis*, was strictly associated with higher altitudes. Although this species was collected only at higher elevations, other collection records reveal that populations of *A. argyritarsis* also occur at lower elevations in KARST (Bertram 1971; unpublished observation). Therefore, any final conclusions about the association of this species with higher altitudes must await additional data.

Habitat Types. A second approach to larval analysis was based on the classification of habitats into habitat types according to their dominant vegetation. With 12 habitat types derived from 73 sampling sites, there were not enough replicates of each habitat type for detailed statistical analysis. However, using a G test of independence, several significant associations were found between mosquito larvae and habitat types. We were also able to rank the habitat types into groups of high, medium, and low densities of larvae. In our previous article (Rejmankova et al. 1992), we pointed out that, in addition to

knowing whether habitats are associated with low, medium, or high larval densities, we also need to know the spatial and temporal extent of habitats to estimate their contribution to mosquito production. For example, habitat types of cyanobacterial mats and submersed-periphyton are in the high larvae-producing group in the dry season, whereas only cyanobacterial mats continue as high producers during the wet season. Evaluating the spatial distribution of individual habitat types in the regions should be a next step in our research effort.

Regions. Certain habitat types are related to specific regions, and they reflect the regional geology, hydrology, water, and soil quality. The MPR provides only two habitat types related to fast-flowing rivers and streams; i.e., rock pools and filamentous algae. The filamentous algae habitat type was not found very frequently in this region, probably because of a very low nutrient content of water. It is highly probable, however, that if streams and rivers from MPR became polluted, they would support more vigorous growth of filamentous algae and would provide a suitable habitat for *A. pseudopunctipennis* larvae. KARST is more diverse than MPR, but the most common habitat type (particularly in the dry season) was filamentous algae with associated populations of *A. pseudopunctipennis*. Populations of *A. albimanus* and *A. crucians* were found rather infrequently in KARST. Diverse fresh and

Table 6. Cross-validation of discriminant functions for *A. pseudopunctipennis* using five randomly selected data subsets

Subset no.	Derived from P/A ^a	Applied to P/A ^a	% Correctly predicted		Coefficients			Cut-off value
			As present	As absent	d ₁	d ₂	d ₃	
1	8/29	6/30	100	100	0.004	-0.025	5.61	2.21
2	6/31	8/28	86	87	0.004	-0.092	24.50	7.73
3	5/32	9/27	78	93	0.005	-0.005	14.29	6.44
4	9/28	5/31	80	94	0.009	-0.120	11.28	3.37
5	7/30	7/29	100	90	0.010	-0.017	8.82	4.33
The whole data set			93	93	0.008	-0.050	12.32	4.79

General form of equation: $Z = d_1(Alt) - d_2(WD) + d_3\arcsin(FA)^{1/2}$, where Alt, altitude (m); WD, water depth (cm); FA, filamentous algae (cover percentage after angular transformation).

^a P, number of sites with species present; A, number of sites with species absent.

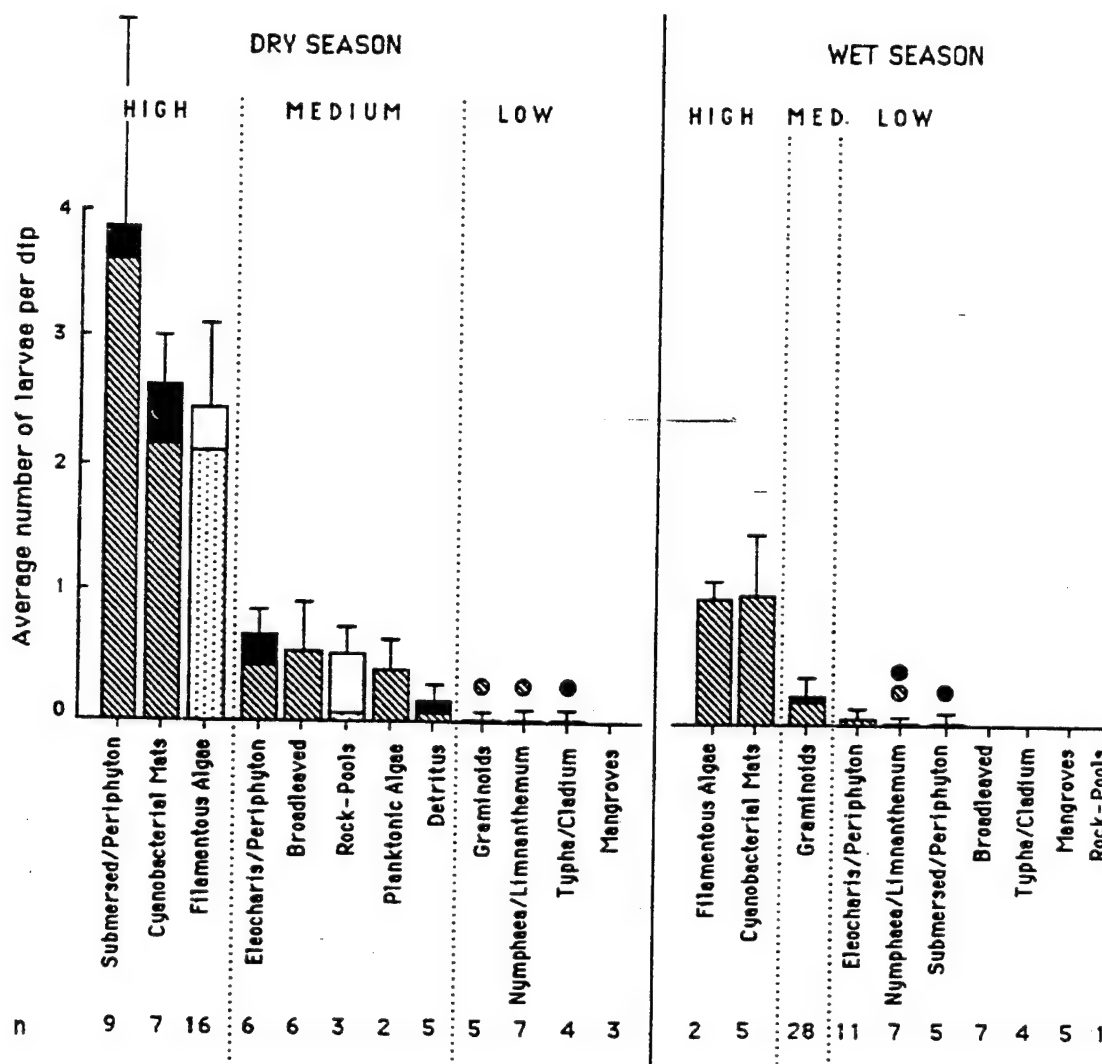


Fig. 3. Distribution of *Anopheles* species expressed as the average number of larvae per dip in individual habitat types. For the habitat description, see text. The number of sampling sites (*n*) belonging to each habitat type is indicated under the figure. Vertical bars indicate the standard error of mean. Wet season, September 1990; dry season, April 1991. For species description, see Fig. 5.

brackish water habitat types supporting both *A. albimanus* and *A. crucians* populations were encountered in CP. The habitat type cyanobacterial mats, which supports *A. albimanus*, was more frequent in CP (brackish). Habitat-types *Eleocharis*-periphyton and submersed-periphyton were common in CP (fresh).

During the wet season, neither *A. argyritarsis* nor *A. pseudopunctipennis* were found, most probably because their habitats were constantly flushed by heavy rains. This was similar to earlier findings in southern Mexico (Savage et al. 1990). Permanent bodies of water generally had the same habitat type and the same *Anopheles* species in both seasons. Larval densities were

generally higher during the dry season than during the wet season; these differences may be related to smaller volumes of water being available in the dry season.

It is interesting that no environmental factors related to water chemistry, such as individual cation or anion concentrations, total suspended solids, or particulate organic matter, were found to be significantly correlated with the occurrence of larvae, except for *A. albimanus* in the wet season. Of all the environmental factors considered, dominant plant growth forms such as filamentous algae, cyanobacterial mats, submersed macrophytes, etc., showed the closest association with the larvae of particular *Anopheles* species.

Table 7. Transition of habitat-types from wet to dry season

Habitat type ^a	Sampled in wet season Total	Transition period from wet to dry season		Sampled in dry season		
		Dried	Contained water	Extant	Added	Total
BC	5	0	5	4	3	7
N-L	7	3	4	3	4	7
S-P	5	3	2	4	5	9
E-P	11	7	4	3	3	6
Br	7	5	2	0	6	6
Gr	28	23	5	2	6	6
T-C	4	4	0	4	1	5
FA	2	2	0	2	2	4
Ma	5	3	2	2	14	16
RP	1	0	1	1	1	3
De	0	0	0	0	5	5
PA	0	0	0	0	2	2

Numbers of habitats belonging to individual habitat types sampled in the wet season, dried out during the transition from the wet to dry season, containing water even in the dry season, added in the dry season, and total sampled in the dry season. Change from one habitat type to another during the transition period is indicated by arrows.

^a BC, cyanobacterial mats; N-L, *Nymphaea-Limnanthemum*; S-P, submersed macrophytes-periphyton; E-P, *Eleocharis interstincta*-periphyton; Br, broadleaved; Gr, graminoids; T-C, *Typha-Cladium*; FA, filamentous algae; Ma, mangroves; RP, rock pools; De, detritus; PA, planktonic algae.

Physical factors (e.g., water depth, water temperature, and oxygen content) were usually marginally correlated with larval occurrence. This makes results of the analyses based on individual environmental factors very similar to those based on habitat types because the habitat types were defined by dominant plant forms.

The data presented here will eventually be used to develop a geographic information system

on the distribution of malaria vectors in northern Belize. The analyses have led to additional questions related to malaria vector ecology: How soon do *A. argyritarsis* and *A. pseudopunctipennis* habitats develop in the dry season? How will the changes in land use (establishment of citrus plantations, increases in human population and migration, etc.) affect distribution and density of mosquito larval populations?

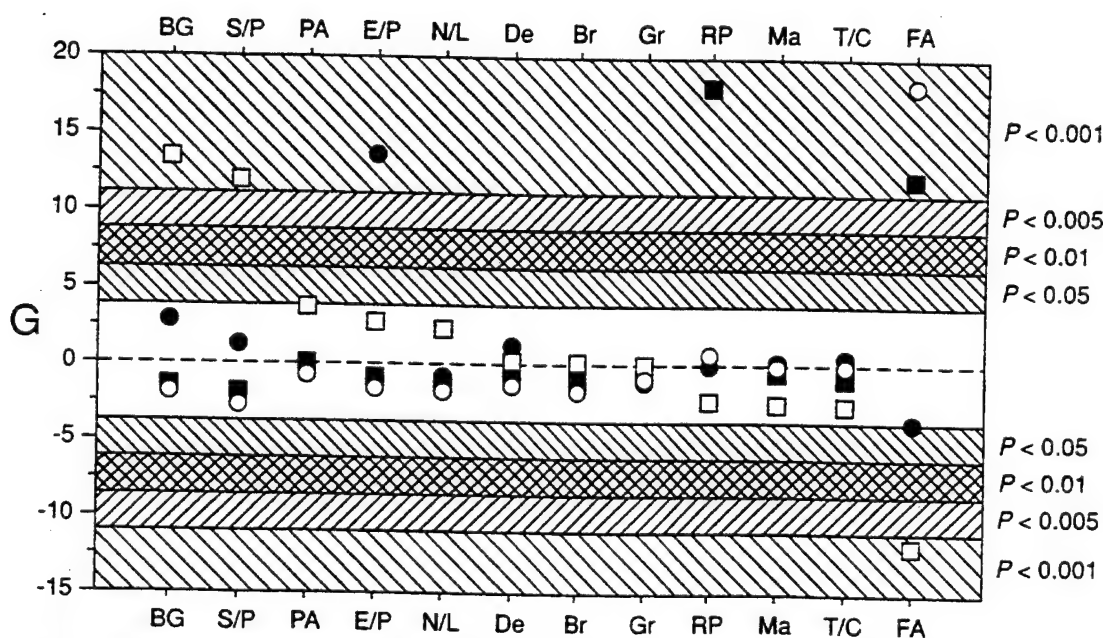
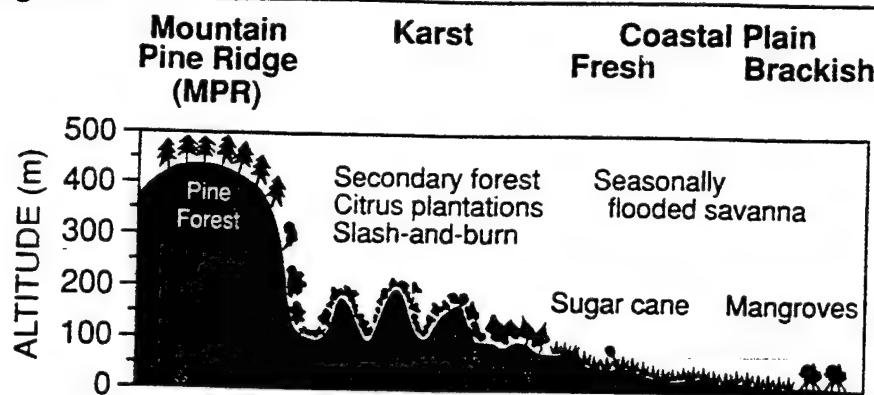
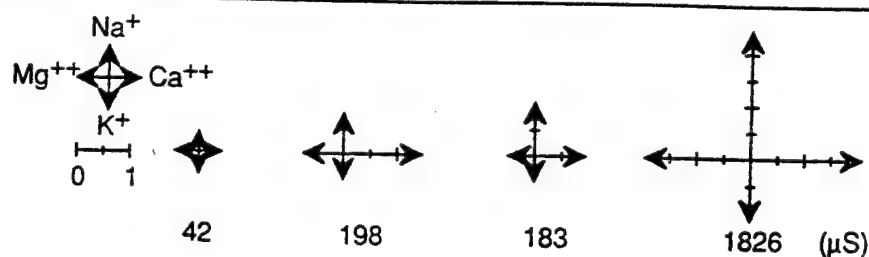


Fig. 4. G test of independence between the habitat-types and *Anopheles* larvae present (Belize, April 1991). BG, Cyanobacterial mats; S-P, submersed macrophytes-periphyton; PA, planktonic algae; E-P, *Eleocharis interstincta*-periphyton; N-L, *Nymphaea-Limnanthemum*; De, detritus; Br, broadleaved; Gr, graminoids; RP, rock pools; Ma, mangroves; T-C, *Typha-Cladium*; FA, filamentous algae. Empty square, *A. argyritarsis*; black circle, *A. crucians*; empty circle, *A. pseudopunctipennis*.

Regions



Cations



Typical Habitats

Rock pools	Filamentous algae	Submersed/Periphyton	Cyanobacterial mats
Filamentous algae	Graminoids	Broadleaved <i>Nymphaea</i> / <i>Limnanthemum</i>	Submersed/Periphyton
		<i>Eleocharis</i> /Periphyton	Graminoids
			Mangroves

Species Distribution

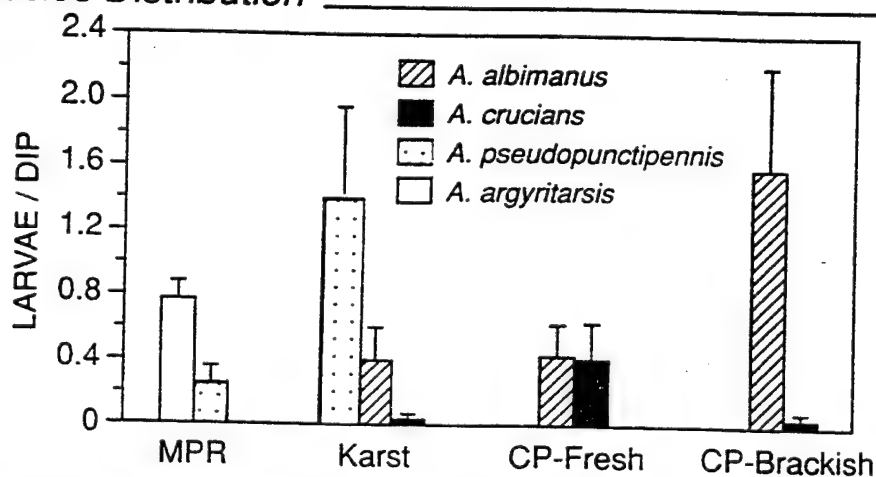


Fig. 5. Mosquito distribution according to physiographic region. Cation concentration is expressed in log mg/liter; numbers below cation diagrams express the specific conductivity. Larval densities for individual species are expressed as the mean number per dip per region; vertical bars indicate the standard error of the mean.

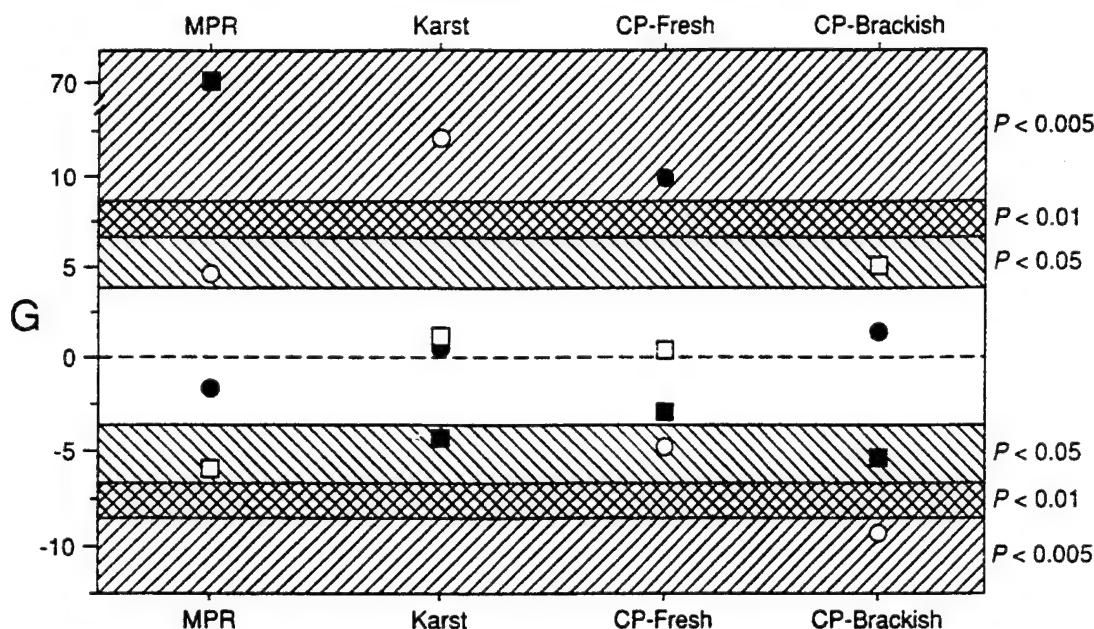


Fig. 6. G test of independence between the regions and presence of *Anopheles* larvae (Belize, April 1991). MPR, Mountain Pine Ridge; Karst, Karst and foothill region; CP (fresh), coastal plain, fresh water; CP (brackish), coastal plain, brackish water. Empty square, *A. albimanus*; black square, *A. argyritarsis*; black circle, *A. crucians*; empty circle, *A. pseudopunctipennis*.

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Appendix 1. List of plant species related to *Anopheles* spp. larval habitats; Belize, wet season, September 1990; dry season, April 1991

	Season ^a	
	Wet	Dry
Emergent		
Gramineae		
<i>Cynodon dactylon</i>	+++	++
<i>Distichlis spicata</i>	++	-
<i>Gramineae</i> sp.	+++	++
<i>Hymenachne amplexicaulis</i>	++	-
<i>Leptochloa</i> sp.	++	-
<i>Panicum</i> sp.	++	-
<i>Paspalum</i> sp.	+++	+
<i>Paspalum virgatum</i>	++	-
Cyperaceae		
<i>Cladium jamaicense</i>	++	++
<i>Cyperus articulatus</i>	+	+
<i>Cyperus ligularis</i>	+	-
<i>Cyperus odoratus</i>	++	-
<i>Cyperus peruvianum</i>	+	+
<i>Cyperus rotundus</i>	++	-
<i>Eleocharis caribea</i>	++	-
<i>Eleocharis cellulosa</i>	++	-
<i>Eleocharis intersticta</i>	+++	+++
<i>Eleocharis mutata</i>	+	++
<i>Eleocharis</i> sp.	++	+
<i>Fimbristylis spadiacea</i>	++	+
<i>Fuirena umbellata</i>	++	+
<i>Rhynchospora barbata</i>	++	-
<i>Rhynchospora cephalotes</i>	+	-
<i>Rhynchospora cyperacea</i>	++	-
<i>Rhynchospora robusta</i>	++	+
<i>Rhynchospora setacea</i>	+	-
<i>Scleria pterata</i>	++	-
<i>Typha domingensis</i>	++	++
Typhaceae		

Appendix 1. Continued

	Season ^a	
	Wet	Dry
Broadleaved		
<i>Bacopa monnieri</i>	++	++
<i>Batis maritima</i>	+	-
<i>Echinodorus</i> sp.	++	+
<i>Heteranthera</i> sp.	-	++
<i>Hydrocotyle</i> sp.	+	-
<i>Hymenocallis</i> sp.	++	++
<i>Justicia</i> sp.	++	+
<i>Lippia nodosa</i>	+	+
<i>Ludwigia octovalvis</i>	+++	++
<i>Pontederia sagittata</i>	++	-
<i>Polygonum</i> sp.	++	++
<i>Sagittaria lancifolia</i>	+	-
<i>Spilanthes</i> sp.	++	++
<i>Wedelia</i> sp.	++	-
<i>Rhizophora mangle</i>	++	++
Floating		
<i>Lemna</i> sp.	+	-
<i>Limnanthemum humboldti</i>	++	++
<i>Nymphaea ampla</i>	++	++
<i>Pistia stratiotes</i>	-	+
Algae		
<i>Filamentous</i>	++	+++
<i>Cyanobacterial mats</i>	++	+++
<i>Periphytic</i>	++	+++
Submersed		
<i>Cabomba</i> sp.	-	++
<i>Chara</i> sp.	++	++
<i>Mayaca fluitans</i>	+	-
<i>Najas guadalupensis</i>	++	+
<i>Potamogeton</i> sp.	-	++
<i>Utricularia cornuta</i>	+	-
<i>Utricularia foliosa</i>	++	++
<i>Utricularia resupinata</i>	++	++
<i>Utricularia purpurea</i>	++	++

+++ , species occurring frequently; ++ , species occurring less frequently; + , species occurring infrequently; - , species not found.

Appendix 2. Detailed Description of Habitat Types

Emergent

Graminoids. Prevalent in the wet season in marshes and seasonally flooded wetlands such as edges of pools and lagoons. Average height above the water surface is 30 cm; often grows to 60 cm; usually not very dense, average cover is 30%. Typical for CP and KARST; fresh waters.

***Eleocharis interstincta*-Periphyton.** A common habitat type in the wet season. Present in most depressions in seasonally flooded savanna, usually forming large uniform areas with plants up to 40 cm tall and covering $\approx 50\%$ of the water. *Utricularia foliosa* as a submersed codominant is quite frequent. This habitat type is less common during the dry season as many habitats become dry. Those dry season habitats with water have senescent *Eleocharis* which is often covered with dense periphyton (Cyanobacteria and Chlorophyta). Typical for CP with fresh or sometimes slightly brackish waters.

***Typha-Cladium*.** Represented by very tall (up to 3 m) and usually very dense (90% cover) emergent macrophytes, mostly *Typha domingensis* or *Cladium jamaicense*; occurring in relatively permanent marshes in both wet and dry seasons and in both fresh and slightly brackish waters.

Broadleaved. Very broad and diverse group of habitats, often containing *Ludwigia octovalvis* as a dominant species. Sometimes low shrubs are present. This, often species-rich, habitat type is generally found on edges of ponds, ditches, and pools and is typical for seasonally flooded areas where aquatic vegetation does not have time to develop. This habitat type is absent in the dry season.

Mangroves. Mostly *Rhizophora mangle* with no other vegetation occurring in salt or brackish waters. This habitat type is present in both wet and dry seasons.

Floating

***Nymphaea-Limnanthemum*.** Floating-leaved macrophytes in more or less permanent fresh

waters of ponds and lagoons. Often relatively dense, large, rigid leaves cover the water surface.

Cyanobacterial Mats. Large dense floating mats (scums) consisting of microscopic benthic Cyanobacteria, known also as blue-green algae (e.g., *Phormidium*, *Lyngbya*). The mats usually develop on the bottom of a water body, then gradually rise to the water surface. Where present, they usually cover large areas. A special microclimate develops in these mats with very pronounced diurnal fluctuations of O_2 , pH, and temperature. More frequent in the dry season but also present in the wet season.

Submersed

Submersed Macrophytes-Periphyton. Several species of submersed macrophytes, such as *Mayaca fluitans*, *Najas guadalupensis*, *Potamogeton lucens*, *Chara* spp., often forming dense populations which may break the water surface. This habitat type develops in mostly permanent water bodies, even though some can grow in seasonally flooded roadside ditches and temporary pools. In the dry season, submersed macrophytes are often densely overgrown with periphytic algae.

Filamentous Algae. Predominantly *Spirogyra* species typical of small rock pools in river beds in both MPR and rivers of KARST. Present mainly in the dry season. During the wet season, this habitat type does not have time to develop because river pools are constantly flushed by heavy rains.

Planktonic Algae. Eutrophic water such as cat-fle ponds; not common and not sampled in the wet season.

Without Vegetation

Rock Pools, No Filamentous Algae. A temporary habitat type present in the dry season in MPR.

Detritus. This habitat type usually develops in small water bodies with fallen leaves and other plant debris.

Prevalence of Hepatitis B Virus Among
Health Care Workers in Belize, Central America

Hepatitis B in Health Workers in Belize

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ABSTRACT

A seroprevalence survey of hepatitis B virus (HBV) markers was conducted among health care workers in Belize to help determine the epidemiology of hepatitis B and to determine if screening before immunization might save vaccine costs. Of the 330 workers tested, 94 (29%) were positive for anti-body to HBV core antigen (anti-HBc) and 3 (1%) had HBV surface antigen. Anti-HBc increased significantly with age from 12% in those 18-24 years to 52% in those ≥ 50 years. The rate was 17% of 48 men compared with 30% of 282 women ($p = .05$). Rates increased with years of medical service and were higher among nurses (69/228; 30%) and domestic workers (15/44; 34%) than among physicians (0/20). Anti-HBc differed significantly among ethnic groups: Mestizo, 4%; Creole, 33% and Garifuna, 57%. Rates differed by district ranging from 3% in a northern district (mostly Mestizo) to 67% in a southern district (mostly Garifuna). Parenteral exposure to hepatitis B through needle stick injuries and blood transfusions was not associated with anti-HBc. Multiple logistic regression analysis confirmed ethnicity, district of residence and age as the best predictors of anti-HBc. Cost analysis suggest that because of regional differences in exposure, testing of health care workers for anti-HBc in Belize and Stann Creek districts in southern Belize before hepatitis B immunization would result in vaccine program cost savings.

INTRODUCTION

Hepatitis B infection is a global occurrence of major health concern. Of adults infected with the hepatitis B virus (HBV), 0.3% to 6% of healthy adults \1-3/ become chronic carriers of HBsAg and among immunocompromised patients, 15% or more become chronic carriers of HBV \4/. Over the past two years, several serological studies of hepatitis B have been conducted in Belize. Seroprevalence for hepatitis B markers has been determined among pregnant women (author's unpublished data), members of the Belize Defence Force (BDF) \5/ and immigrant agricultural workers in the Stann Creek district \6/. To date, no epidemiological data is available for the prevalence of the hepatitis B virus exposure among the health care workers of Belize.

Several studies have indicated that hepatitis B infection in health workers is an occupational risk due largely to percutaneous and mucosal exposure to blood and body fluids \7/. The present study was conducted to determine the prevalence of hepatitis B markers in the health care population with a view toward the introduction of a rational, cost-effective vaccine program that would ultimately minimize the occupational transmission of hepatitis B virus.

MATERIALS & METHODS

The study was approved by the Human Use Review Committees of Belize and the Uniformed Services University of the Health Sciences. Between the months of July and September 1993, the staff from the hospitals and public health centers in the six districts of Belize were invited to participate. After signed informed consent was obtained, a short questionnaire requesting demographic data and selected

medical history was administered to all the volunteers. In order to maintain patient confidentiality, a coded identification number was assigned to a 12 ml venous blood sample drawn from each participant and to the corresponding questionnaire. At the Belize City Hospital, workers on all shifts were invited to participate, while in the district hospitals, only workers on the day shift were recruited.

Laboratory evaluation on sera was performed using Enzyme-linked immunosorbent assay (ELISA) for antibody to hepatitis B core antigen (ETI-AB-COREK from Sorin Biomedica, Saluggia, Italy). HBsAg was detected using Auszyme (Abbott Diagnostics, Abbott Park, IL). Individual results were confidentially distributed to all volunteers and those with HBsAg reactivity were advised to undergo repeat testing after 6 months.

Statistical Analysis

Chi-square analysis was used to determine association of collected variables with anti-HBc. Chi-square for trend was used to evaluate age and years of service for association with anti-HBc. Stepwise multiple logistic regression analyses were performed using the statistical program BMDPLR (BMDP Statistical Software, Los Angeles, CA). Only variables which were statistically associated with anti-HBc were entered into the multiple logistic regression model. Health care workers were coded into 4 categories: nurses, domestic workers, doctors, and other paramedical personnel.

To determine whether vaccine program costs would be saved by testing for anti-HBc before vaccination, a formula to include various cost elements was devised similar to one used to evaluate

prevaccination testing for hepatitis A \8/. Total cost of screening and vaccination of only those susceptible to hepatitis B included: Labor cost for venipuncture and lab testing (\$6.00/hr) + cost of test kits for anti-HBc (\$4.00 per test) + travel costs to each district (including gas, per diem and overnight lodging) + vaccine costs (\$27/person for Engerix B, SmithKline Beecham) to immunize only those susceptible + vaccine administration costs to each susceptible person (estimated at \$5.00/person to administer 3 doses). The cost without screening included the vaccine costs for all HCW plus administration costs for all HCW. Average cost per person were obtained by dividing the total costs by the number of health care workers studied.

Three scenarios were evaluated. The first was the actual cost of the study including actual travel costs and laboratory testing that repeated all samples reactive for anti-HBc in duplicate. In the second scenario all reactive samples are repeated only once. In the third scenario single repeat of reactive samples was assumed and travel costs were eliminated assuming that HCW would have their blood drawn by the local hospital lab and the samples would be shipped to the testing lab in Belize City in bulk.

RESULTS

Of an estimated 788 health care workers (HCW) in Belize, a total of 330 (42%) volunteered for the study. All completed a questionnaire and provided a blood sample. Of the participants, 85% were female and 15% were male. The proportion of HCW sampled in each district was highest in Belize (47%) and Orange Walk districts (48%) which were more easily accessed, but was somewhat lower in

Corozal (21%), Cayo (35%) and Stann Creek districts (37%). Almost half of the 50 HCW in the most distant district, Toledo, were studied by flying in and spending 2 days doing collections. The mean age was 33 years and ranged from 18 to 66. Self-reported ethnic groups were distributed as follows: 47% Creole, 22.5% Mestizo (of Spanish and Mayan origin), 16% Garifuna (of African and Carib Indian ancestry), 3% indigenous Indian (Ketchi and Mopan Maya), 2% East Indian, 0.5% Lebanese and 9% in-transit workers of other descent (Cuban, Guyanese, Guatemalan). Longevity of health service work varied from a few weeks to more than 20 years, the largest group (37%) serving from 0 to 5 years.

Antibody to hepatitis B core antigen (anti-HBc) was detected in 94 health workers (30%). Hepatitis B surface antigen (HBsAg) was detected in 3 (0.9%). The seroprevalence of anti-HBc was 31% in females compared with 17% in male health workers, ($p = .05$) (Table 1). The mean age of persons with anti-HBc was higher (38.4 ± 9.6 years), compared with those without anti-HBc (33.2 ± 9.4 years) ($p < .001$ by t-test). Anti-HBc rates increased significantly with age from 12% in the 18-24 year age range to 52% in those 50 years and above, ($p = .001$).

Significant differences were also noted in the prevalence of anti-HBc by ethnicity and by district. The lowest prevalence of anti-HBc reactivity was found in those of Mestizo ethnicity (4%). Serologic evidence of exposure to hepatitis B was found most commonly among Garifuna (57%), a significantly higher prevalence rate than for Creole (33%) and East Indian (28%) ethnic groups, ($p = .001$). No anti-HBc was found among the few Mayan and Lebanese studied. The lowest

anti-HBc seroprevalence was 3% in the northern district of Orange Walk (mostly Mestizo) while the highest was 67% in the southern district of Stann Creek (mostly Garifuna) ($p < .001$).

An increase in anti-HBc was noted with increasing years of health care service ranging from 20% of those serving less than 5 years to 40% of those in service more than 20 years, ($p = .07$). Significantly higher rates of anti-HBc were observed among nurses, who comprised (69%) of the HCW studied, as well as domestic workers and other para-medical personnel compared with physicians. No evidence of hepatitis B infection was found in the 20 physicians studied compared with rates $\geq 30\%$ among other personnel.

Even though 30% of the HCW had antibody to hepatitis B, only 2.4% gave a history of physician documented hepatitis, 4% gave a history of symptoms of hepatitis, and 14% a history of hepatitis in their families. Anti-HBc was not more common in any of these groups (Table 2). Neither receipt of blood transfusions, exposure to needle sticks, medical injections, or IV fluids was associated with evidence of hepatitis B markers among the study population. The prevalence of anti-HBc was not more common in persons who admitted such reported risk factors as tattoos, pierced ears or blood letting (an invasive procedure performed by a healer to cure certain diseases in some cultures).

Multiple logistic regression analyses revealed 3 variables to be independently associated with anti-HBc: district, ethnicity and age (Table 3). After adjustment for the age and ethnicity, workers in Belize and Stann Creek District had a significantly higher likelihood of having anti-HBc relative to health care workers in Orange Walk

district. Likewise, Creole, Garifuna, and East Indians were 5.3, 15.0, and 11.2 times more likely to have anti-HBc, respectively, compared with health care workers who are of Mestizo ethnicity. The model also predicts that for each year of increasing age, one is 1.06 times more likely to have anti-HBc. For example, a person 30 years of age is 1.74 times more likely to have anti-HBc than a person 20 years of age.

The cost of screening and vaccinating in the three southern districts where the prevalence of anti-HBc exceeded 30% was compared with the estimated costs of vaccinating all HCW. Table 4 indicates that the average cost per health care worker for screening and vaccination of only susceptible HCW compared with vaccination of all workers. The first scenario is the way this study was performed and repeating all samples reactive for anti-HBc in duplicate. This method would result in minimal cost savings in Belize District where the prevalence was 32% but increased costs in Toledo District because travel costs to this southernmost district are high. Screening would result in modest cost savings (2.7% per person) in Stann Creek District where the prevalence of anti-HBc was 67%. In the second scenario, if sera initially reactive for anti-HBc were retested only once, larger program savings (4.1% - 14.2%) would result in Belize and Stann Creek districts. In the third scenario where travel costs were eliminated, cost savings were increased in Stann Creek district to 21.6% but little increased savings would accrue in Belize District (4.4%) where travel costs were minimal.

DISCUSSION

In this study, a high rate of previous exposure to hepatitis B was demonstrated in health care workers. However, the anti-HBc prevalence of 30% in health care workers compares with 31% among the Belize Defense Force (BDF) \5/. If only female health care workers 18 to 35 (mean 27.9) years of age are considered, the rate of 21% appears higher than the rate observed among women of the same age range (mean age 24.4 years) attending prenatal clinics in Belize (14%) ($p = .02$) (authors unpublished data). As with other studies in Belize, anti-HBc seroprevalence was associated with ethnicity, district and age \5, authors unpublished data/. A case control study with controls matched for age, sex and ethnicity would be necessary to determine if the prevalence of HBV is higher among HCWs.

Logistic regression analyses in the present study and in a previous study of women attending prenatal clinics in Belize confirm ethnicity as a strong predictive factor for HBV exposure in Belize. Persons of Garifuna ethnicity had significantly higher rates of anti-HBc than other ethnic groups. The rate among Garifuna health care workers was 57% compared with 41% among Garifuna attending prenatal clinics \authors unpublished data/ and 56% among Garifuna (mostly men) in the Belize Defence Force \5/. The reason(s) for the high prevalence of anti-HBc among Garifuna is unknown. Reported rates of anti-HBc among Africans and Carib Indians have been high \9, 10/. The rate among Creole health care workers (33%) was similar to Creole in the BDF (30%) and among prenatal women (19%). Mestizos have consistently had one of the lowest rates of anti-HBc:

4% among health care workers, 5% among the BDF, and 8% among prenatal women.

Ethnicity is significantly associated with hepatitis B in other countries as well. Black health care workers in Zimbabwe were noted to have higher rates of anti-HBc than other ethnic groups \11/. Rates of anti-HBc among health care workers in the United States have ranged from 33-53% among Asians, 23-32% among those of African descent, and 9.4-10.2% among white hospital personnel in two California hospitals \12/. Studies are on going in Belize to help define the epidemiology of hepatitis B among different ethnic groups.

In the present study, district of work was also strongly predictive of anti-HBc. After controlling for age and ethnicity, the odds of having anti-HBc among health care workers in the Stann Creek district were 20 times that of health care workers in the Orange Walk district. Garifuna comprise 36% of the population of Stann Creek district (1991 census) and 75% of the health personnel in Stann Creek district who were studied. Rates of anti-HBc in members of the BDF from Stann Creek and women attending prenatal clinics were also high. In addition to high rates among Garifuna and Creoles in the Stann Creek district, rates of anti-HBc of 70% were found among Central American immigrants on banana plantations in this district \7/. In the United States, hepatitis B rates in the general population \13/ and in health care workers vary greatly by region from 1.7% in Minnesota and Utah \14/ to 40% in one hospital in California \12/.

Increasing age as a predictive factor for hepatitis B has been noted in other studies in Belize and in health care workers in other

countries \15/. It is unknown if this is a cohort effect or, more likely, cumulative exposure to hepatitis B with increasing age. Age is a better predictor of anti-HBc than years of medical service according to the logistic regression model.

By multiple logistic regression, type of medical occupation in Belize was not predictive of anti-HBc. The lower rate observed among medical officers is likely the result of ethnic background since most are Mestizo and Creole, groups with lower overall rates. Incidence rates of hepatitis B in health care workers in the United States have been highest in those that work in large tertiary care hospitals, especially on dialysis units and in the laboratory. Rates among physicians and non-dialysis nurses are much lower \16/. Hospitals in Belize are small and hemodialysis is not performed.

As in the studies of health care workers in Zimbabwe and Jamaica, the present study did not demonstrate an association of hepatitis B with exposures such as needlestick injuries. Apparently, much hepatitis B exposure in Belize occurs outside the health care setting. However, other studies have documented well the increased occupational risk of hepatitis B in health care workers \16, 17/. In fact, since completion of the study, one health care worker who was found to be susceptible to hepatitis B was hospitalized with acute hepatitis B thought to be occupationally acquired.

The most important means of prevention of hepatitis B and other blood-borne pathogens such as HIV-1 has been through increased attention to hospital infection control and the use of universal precautions \18, 19/. These methods are thought to be responsible for a 12-fold decrease in occupationally acquired hepatitis B in

Minnesota health care workers to a rate comparable to that of the general population of Minnesota before hepatitis B vaccine was available \16/. Proper disposal of blood contaminated materials, sharp instruments and needles, as well as the use of gloves, will provide the greatest benefit against all blood-borne infectious agents. The introduction of hepatitis B vaccination among health care workers may further reduce hepatitis B infection as noted in a previous study \19/.

Use of screening strategies to detect anti-HBc as an indication of previous exposure before immunization have proven useful in Belize \5, 6/ and in the United States \12, 18, 20/. Cost analysis studies suggest that vaccination after screening is cost effective in groups with a prevalence of HBV markers in the range of 22-39% \12/. In Belize, screening health care workers prior to immunization in Belize and Stann Creek districts which have the highest rates of anti-HBc would reduce vaccine program costs.

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Table 1

Anti-HB core Rates by according to Demographic Data of Health Care Workers

	No. Positive	No. Tested	% Positive	Odds ratio	Conf. Int.	P value
Gender						.05
Male	8	48	17	1.0		
Female	86	282	31	2.19	0.94, 5.32	
Age (Years)						0.001
18-24	6	50	12	1.00		
25-29	12	56	21	2.00	0.62, 6.64	
30-34	18	75	24	2.32	0.78, 7.17	
35-39	17	52	33	3.56	1.16, 11.41	
40-49	26	68	39	4.54	1.57, 13.76	
≥ 50	15	29	52	7.86	2.27, 28.43	
Ethnicity						<0.001
Mestizo	3	74	4	1.00		
Temporary	8	30	27	8.61	1.84, 45.41	
Foreign Workers						
East Indian	2	7	28	9.47	0.85, 102.72	
Creole	51	155	33	11.61	3.31, 48.55	
Garifuna	30	53	57	30.87	7.92, 140.8	
Mayan	0	10	0	0.00	0.00, 18.96	
Lebanese	0	1	0	0.00	0.00, 591.63	

District						<0.001
Orange Walk	1	35	3	1.00		
Cayo	6	40	15	6.00	0.65, 139.47	
Corozal	2	16	13	4.86	0.30, 148.26	
Belize	61	191	32	15.95	2.26, 320.56	
Toledo	8	24	33	17	1.84, 394.34	
Stann Creek	16	24	67	68	7.30, 1592.95	
Years of Service						0.07
0-5	24	121	20	1.0		
6-10	23	77	30	1.72	.84, 3.51	
11-15	20	63	32	1.62	.77, 3.38	
16-20	12	31	39	2.2	.87, 5.51	
≥ 20	15	32	40	3.07	1.26, 7.51	
Occupation						.03
Physicians	0	20	0	0.00	0.00, 0.59	
Nurses	69	228	30	1.0		
Domestic	15	44	34	1.19	.57, 2.48	
Other*	10	38	27	.82	.35, 1.89	

* Includes Laboratory technicians, 2; Attendants, 13; Paramedics, 4; Dental, 6; Clerical, 4; Public Health, 3; Radiology, 2; Pharmacy, 4

Table 2. Epidemiologic Risk Factors by Presence of Anti-HBc and odds ratio compared with those without variable

	No. with anti-HBc	No. with variable	% with anti-HBc	Odds Ratio	P Value
Hepatitis in family	14	46	30	1.1	.75
Hepatitis in past	3	8	38	1.5	.56
Symptoms of hepatitis	4	13	31	1.1	.85
Blood transfusion	21	67	31	1.2	.56
Needlestick injury	69	229	30	1.3	.31
Injections	83	294	28	.9	.77
Intravenous fluids	67	224	30	1.3	.40
Tattoos	2	21	10	.2	.08
Pierced ears	83	280	30	1.5	.27
Blood letting	1	5	20	1.7	.93

TABLE 3. Estimated Odds Ratio and 95% Confidence Intervals (C.I.)
from the Stepwise Logistic Regression Model

	Odds Ratio	Lower 95% C.I.	Upper 95% C.I.
District *			
Orange Walk	1.0	-	-
Belize	11.1	1.3	94.8
Corozal	7.7	0.5	109.0
Cayo	4.8	0.5	47.8
Toledo	5.8	0.6	57.7
Stann Creek	20.8	2.1	207.0
Ethnicity **			
Mestizo	1.0	-	-
Creole	5.3	1.8	16.0
Garifuna	15.0	4.2	53.4
East Indian	11.2	1.4	89.1
Other	3.4	0.7	15.4
Age	1.06	1.03	1.09

* Relative to Orange Walk

** Relative to Mestizo

Table 4. Average cost per person of screening for anti-HBc and vaccination of susceptible health care workers with hepatitis B vaccine compared with vaccinating all health care workers in three districts in Belize.

District	Scenario One*	Scenario Two**	Scenario Three***	Vaccinate all
Belize	\$31.95	\$30.67	\$30.58	\$32.00
Stann Creek	\$30.13	\$27.46	\$25.08	\$32.00
Toledo	\$49.00	47.67	\$33.19	\$32.00

* Travel to district to collect blood; repeat all samples reactive for anti-HBc in duplicate

** Travel to district to collect blood; repeat all samples reactive for anti-HBc once.

*** Do not travel to districts; repeat all reactive samples once.

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The Environment, Remote Sensing, and Malaria Control

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INTRODUCTION

There is increasing interest in environmental protection to conserve ecological processes, preserve genetic diversity and sustain use of natural ecosystems. In public health, concern for the environment focuses on contributions of the environment to emergence of diseases, including the arthropod-borne diseases. In the case of environmental protection, the goal is to prevent human intrusions and modifications of natural environments for the primary purpose of living resource conservation. In efforts to protect public health, the primary goal should be to prevent human intrusion and modification of pristine environments in order to protect human populations from extensions of pathogens from natural foci^a of disease.¹ In each case, protection of the natural environment is emphasized. The International Council of Scientific Unions and the International Geosphere-Biosphere Program emphasize the need for global data and information systems to address environmental and conservation concerns.² Among health professionals, the need is also recognized for global monitoring and information systems on environmental issues and emerging diseases.^{3,4}

Improved communications and space-science technologies, such as remote sensing, offer hope of new, more holistic approaches to surveying, monitoring and controlling the arthropod-borne diseases. The promise of these technologies has surfaced at a time when global and national efforts at vector and disease control are faltering, for example *Aedes aegypti* and malaria, respectively.

REMOTE SENSING TECHNOLOGY

Remotely sensed data of various types and of various scales can be used to elucidate and predict the temporal and spatial distributions of disease

^a Natural foci of disease are environments, undisturbed by human activities, where vectors, pathogens and recipients of infection (vertebrate hosts) reside.

vectors. Low resolution data (*e.g.*, US National Oceanic and Atmospheric Administration satellite data) have been used to plot normalized difference vegetation indexes (NDVIs) indicative of where or when vectors of Rift Valley fever or African trypanosomiasis might occur^{5,6} (see FIG. 14 in REMOTE SENSING SECTION). Such low resolution data are useful for general studies of broad zones or regions. Higher resolution data from Landsat or SPOT satellites can be used to study the temporal and spatial distributions of disease vectors in association with small earth surface features, rice fields, villages, rivers, *etc.*⁷⁻⁹ (see FIG. 13 in REMOTE SENSING SECTION). Work with high resolution data can take advantage of increasingly specific knowledge of the vector-environmental associations. In other words, information on how different surface features will impact the local presence and abundance of the vector species can be used in interpretations of satellite data. Consequently, background studies on the relationships of the environment to disease-vector-host associations are needed for appropriate use of remote sensing technology.

Background studies should be designed to detect, identify and analyze the environmental determinants of disease vectors in the real world; define the scale for detecting, identifying and analyzing environmental determinants with satellite data; and finally, validate analyses of satellite data with *in situ* (ground truth) data (FIG. 1). The results of these studies can be used to

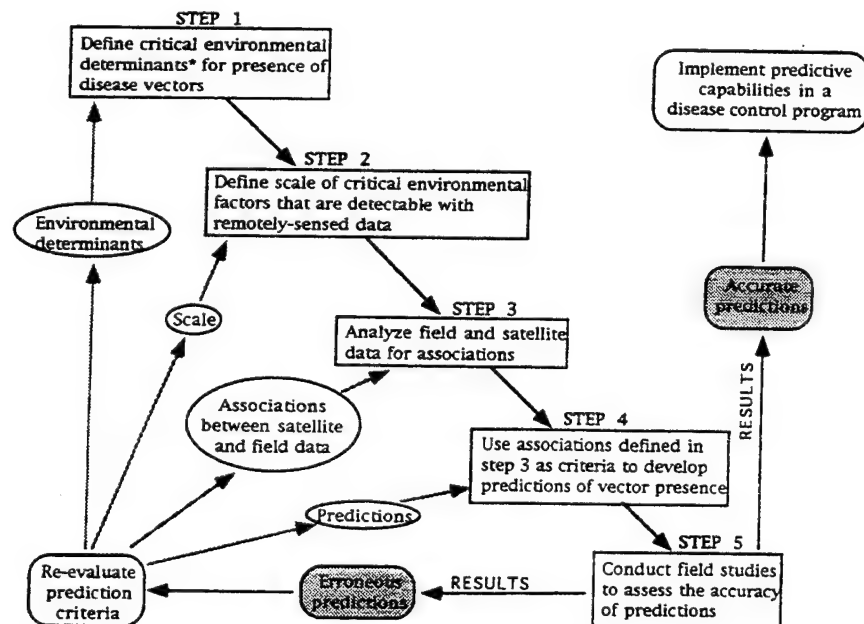


FIGURE 1. A paradigm of required studies leading to applications of remote sensing data to predict the spatial distributions of disease vectors. * Indicates earth surface features, *e.g.*, rivers, ponds, pastures, forest edge that influence the presence and abundance of disease vectors.

prepare and execute regional schemes of disease and vector control, predict future events, and plan and develop projects for preventing disease risks. The background studies' data are derived from field studies, and should be amenable to computer manipulation. For many other categories of background data, remote sensing is the only cost-effective means of acquiring data to study, monitor, and hopefully, contain or mitigate existing and emerging vector and disease problems.

Arthropod-borne diseases in particular are strongly associated with certain environmental conditions. Indeed, the principal environmental conditions, such as temperature and rainfall, regulate the geographical and seasonal distribution of many arthropod-borne diseases. Recognition of the interactions between humans, disease vectors and the environment date back far into the history of infectious disease research. The relationships were formalized in Pavlovsky's research in Russia and his writings on landscape epidemiology.¹ Pavlovsky noted that arthropod-borne disease exists when there are specific climate, vegetation, soil, and favorable microclimate in the places where vectors, donors and recipients of infection take shelter. Furthermore, he noted that disease circulation takes place only when the environmental conditions are favorable. Examples of diseases that are regulated by environmental conditions include leishmaniasis, African trypanosomiasis, malaria and others.

REMOTE SENSING TECHNOLOGY AND MALARIA CONTROL

Today malaria continues to be the leading cause of morbidity and mortality in humans throughout the tropics, with an estimated 270 million people affected. Despite efforts to eradicate the disease, it has made a dramatic resurgence within the last 20 years. Furthermore, morbidity and mortality from malaria are at almost unprecedented levels.¹⁰

Application of remote sensing to study and assist in the control of malaria and other arthropod-borne diseases is the subject of a National Aeronautics and Space Administration (NASA)-sponsored study.¹¹ The NASA project^b is designed to demonstrate the use of remote sensing technologies to develop predictive models of malaria vector abundance on a local to regional scale.

Human malaria is an anthroponosis, as such it is only found in association with human populations. It is not an emerging disease or a disease associated with natural foci, as defined by Pavlovsky.¹ Indeed, malaria foci only occur when humans, vectors and the malaria parasites occur together. However,

^b Participants in the NASA-sponsored project include the following individuals and organizations: Louisa Beck, Sheri Whitney and Mike Spanner, NASA Ames Research Center, Moffett Field, California; Eliska Reimankova and Robert Washino, University of California, Davis, CA; Mario H. Rodriguez, Americo Rodriguez and Juan Hernandez, Centro de Investigacion de Paludismo, Tapachula, Mexico; Jack Paris, California State University, Fresno, CA; Carl Hacker, School of Public Health, University of Texas in Houston; LLewellyn J. Legters, Donald Roberts and Sylvie Manguin, Uniformed Services University of the Health Sciences, Bethesda, MD.

since malaria is transmitted by *Anopheles* mosquitoes, human malaria is associated with natural environments to the same extent that the mosquito vectors are associated with natural environments.

The NASA project was conducted in three phases and we are currently in phase III. The first phase was conducted on population abundance of *Anopheles freeborni* in rice fields of the Central Valley of California. The first phase of work tested the concept that remote sensing and ground surveillance data incorporated into a geographic information system (GIS) could be used to predict the temporal and spatial occurrences of *A. freeborni* larval populations. In brief, we found that early developing rice fields (defined by remotely sensed data) in closest proximity to livestock pastures produced greater densities of larvae than more slowly developing fields located further from pastures. The remote sensing reflectance data and GIS measurements of rice field-distances from pastures were adequate to predict nearly 90% of the high-producing fields almost 2 months before peak densities of *Anopheles* larvae.⁷

Phase II was conducted in the state of Chiapas, Mexico in collaboration with the Centro De Investigaciones de Paludismo in Tapachula. The general approach for Phase II studies has been described previously.¹² The first priority for Phase II studies was to improve understanding of the biology of the primary malaria vector species *A. albimanus*, on the Coastal Plain. Initial research was directed at defining the relationships of *A. albimanus* larval abundance with a variety of environmental variables. The relationships of the dominant aquatic plants with the presence and abundance of larvae were particularly germane to our research interests. In phase II research, habitats were defined by dominant plants; habitats were then clumped (by use of cluster analysis) into habitat-types. Habitat-types were then used to characterize the larger vegetation units. The vegetation units, unlike habitats and habitat-types, were generally discernible with remotely sensed data. A habitat was defined as a body of water with a dominant vegetation, for example *Eichhornia*. The habitat-types were defined strictly on botanical and limnological parameters. Analysis of variance was employed to investigate relationships among habitat-types and the density of larvae. These studies showed that selected growth forms of plants contributed positively, neutrally, or negatively to the presence and abundance of *A. albimanus* larvae. Finally, the larger vegetation units, which were detectable with remotely sensed data, were characterized with certain mixes of habitat-types and larval densities.¹³⁻¹⁶

In conducting current phase II research, a GIS was developed that includes digitized map attribute data, geolocated and classified remotely sensed data, and field surveillance data. A standardized program of surveillance with UV-updraft light traps and ground verification of breeding sites surrounding villages was used to obtain the *in situ* data. The satellite data are used to identify villages and classify vegetation units surrounding villages. Relying on previously defined associations of vegetation units with *A. albimanus* larval abundance, remotely sensed data were used to define the associations of vegetation units and individual villages with high or low densities of *A. albimanus* mosquitoes.⁸

TABLE 1. Studies Conducted on the Uses of Remote Sensing to Predict the Spatial Distribution of *Anopheles* Mosquitoes in California, Mexico and Belize

Studies	California	Mexico	Belize
Step 1: Define environmental determinants		X	X
Step 2: Define scale		X	X
Step 3: Define associations between remote sensing and field data	X	X	
Step 4: Develop predictions about spatial distribution of vectors		In process	X
Step 5: Assess accuracy of predictions		In process	X

Source: Wood *et al.*,⁷ Beck *et al.*,⁸ and Roberts *et al.*⁹

In a separate program of research,⁶ remote sensing and cartographic data were employed to predict localities of high and low malaria vector densities during the dry season along the Hummingbird Highway in Belize (see color FIG. 13). Predictions were based on environmental criteria derived from two years of field studies. Predictions were developed by remote sensing specialists with no previous experience in Belize and no field data. The criteria for predictions related to presence of waterways, elevation, amount of forest between houses and selected waterways, and presence of humans. Once predictions were developed, field surveys were conducted in April and May, 1993 to verify presence and abundance of vectors. This blind test was the first time that remotely sensed data have been used to develop highly accurate prospective predictions about the spatial distributions of malaria vectors.⁹ Although the SPOT multispectral satellite data employed in this test were from 1990, still the data interpretations relating to ground cover and sites of houses and human activities were generally accurate. While single houses with thatch roofs could not be detected, there were other characters indicative of human activities, and thus presence of humans.

Studies in California, Mexico and Belize differed in terms of approaches as defined by the paradigm in FIGURE 1 (TABLE 1). Field work conducted by Dr. Robert K. Washino on *A. freeborni* in California precluded the need for preliminary field studies (step 1). Additionally, the determination of scale for integrating remote sensing and field data (step 2) was fixed by size of the sampling unit, *i.e.*, rice field. Studies were performed to define associations between spectral and *in situ* data;⁷ but no tests of predictions were performed (steps 4 and 5). In Mexico, extensive field studies (step 1) were conducted to define associations of *A. albimanus* with environmental determinants.¹³⁻¹⁶ These studies were followed by a detailed study of associations between remotely sensed and *in situ* data (step 3).⁸ Based on the latter study, predic-

⁶ Eliska Reimankova, University of California, Davis, CA; Jack Paris, California State University, Fresno, CA; LLeueLLyn J. Legters, Donald Roberts and Sylvie Manguin, Uniformed Services University of the Health Sciences, Bethesda, MD; Jorge Polanco, Ministry of Health, Belize City, Belize.

tions of vector abundance in different villages will soon be developed (step 4) and tested with field data (step 5). For studies in Belize, extensive field studies (step 1) were conducted to define associations of *A. pseudopunctipennis* populations with environmental determinants.^{15,17} Specific environmental determinants that were detectable with multispectral satellite data were identified. Since we were very confident of the predictive power of these environmental determinants for presence of *A. pseudopunctipennis* mosquitoes, no study of associations between remotely sensed and *in situ* data (step 3) was conducted. Consequently we were able to skip step 3 and complete steps 4 and 5 by developing predictions and testing the predictions with field survey data.

SUMMARY

Results of studies in California, Mexico and Belize demonstrate the value of remote sensing technology for studying vector-borne diseases. These studies have also shown that it is necessary to fully define the environmental factors associated with the presence of vectors and disease transmission, and to be able to detect these environmental factors with image data. These studies, and other published reports,^{5,6} are demonstrating many potential uses of remotely sensed data in managing and targeting vector and disease control measures.

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PRELIMINARY OBSERVATIONS ON THE CHANGING ROLES OF MALARIA VECTORS IN SOUTHERN BELIZE¹

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S. MANGUIN,² J. POLANCO¹ AND L. J. LEGTERS²

ABSTRACT. A survey for larval and adult *Anopheles* mosquitoes was conducted in Toledo District of southern Belize during August–September 1992. We surveyed for larvae in 145 habitats and conducted paired indoor–outdoor collections of adult mosquitoes landing on humans at 6 houses. In 1940–41, Kumm and Ram reported *Anopheles darlingi* females to be the most common *Anopheles* mosquitoes inside houses and reported no specimens of *Anopheles vestitipennis* in southern Belize. In our 1992 survey we found no *An. darlingi* mosquitoes either as adults or larvae. More *An. vestitipennis* females were captured indoors than outdoors, whereas most *Anopheles albimanus* and *Anopheles apicimacula* females were captured outdoors. All 3 species were represented occasionally in 145 collections of larvae from diverse habitats. *Anopheles vestitipennis* now appears to be a potentially important vector of malaria during the wet season in Toledo District.

The presence of *Anopheles darlingi* Root in Belize was first reported by Komp in 1940. The identity of Komp's original *An. darlingi* specimens was recently verified by Linthicum (1988). In 1941, Kumm and Ram documented the occurrence of house-frequenting populations of *An. darlingi* in the Toledo and Stann Creek districts of Belize (Fig. 1). Kumm and Ram relied heavily on searches of houses for resting mosquitoes as their primary survey method. *Anopheles darlingi* was found in 3 of 7 localities surveyed in Toledo District and in 5 of 7 localities in Stann Creek District. The larvae of *An. darlingi* were also collected in both districts. *Anopheles vestitipennis* Dyar and Knab was not collected as adults or larvae in Toledo District, but was collected as adults at 3 of 7 localities and as larvae in Stann Creek District. *Anopheles albimanus* Wiedemann was the most widely distributed species, being present at 9 of 14 localities from both districts. Larvae of *An. albimanus* were also collected in both Toledo and Stann Creek districts. Malaria sporozoites were found in the salivary glands of *An. darlingi* and *An. vestitipennis*, but not *An. albimanus*. *Anopheles darlingi* was the dominant indoor anopheline, representing more than 70% of the anophelines caught indoors in rural areas.

The Kumm and Ram survey was conducted more than 50 years ago, prior to the use of DDT in the malaria control program in Belize. Since their survey, no comparable data have been published for the Toledo District. Thirty years later, Bertram (1971) did not encounter a single specimen of *An. darlingi* in an extensive survey of adult mosquitoes in northern Belize, including areas of Stann Creek District. Although few collections have been conducted in Belize, the last documented appearance of *An. darlingi* in Belize was at Serra de Aqua in June 1946 (Linthicum 1988).

We initiated a malaria vector research program in Belize in 1990 and conducted extensive larval surveys in northern Belize, including Corozal, Orange Walk, Belize City, Cayo, and Stann Creek districts. No *An. darlingi* or *An. vestitipennis* larvae were collected in those surveys (Rejmankova et al. 1993). In a recent wet season survey in Toledo District we included nighttime, paired indoor–outdoor landing collections from humans to increase the likelihood of detecting the presence of *An. darlingi* and *An. vestitipennis*. These collections were performed by capturing mosquitoes as they landed on the exposed legs and feet of 2–4 collectors. Paired indoor–outdoor collections, using 1–2 collectors per indoor or outdoor site, were conducted one evening at each of 6 localities from 1830 to 1915 h. Based on past experience (Roberts et al. 1987), we expected the sunset interval to be a period of peak *An. darlingi* host-seeking activity. After completing the survey we learned that Rivera-Nunez (1990^a) recently reported a sunset peak (1800–

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^a Rivera-Nunez, L. A. 1990. Algunos aspectos de comportamiento de *Anopheles darlingi* (Diptera: Culicidae) de la Ceiba, Atlantida, Honduras. Thesis. Maestria en Entomologia, Universidad de Panama, Panama City, Panama.

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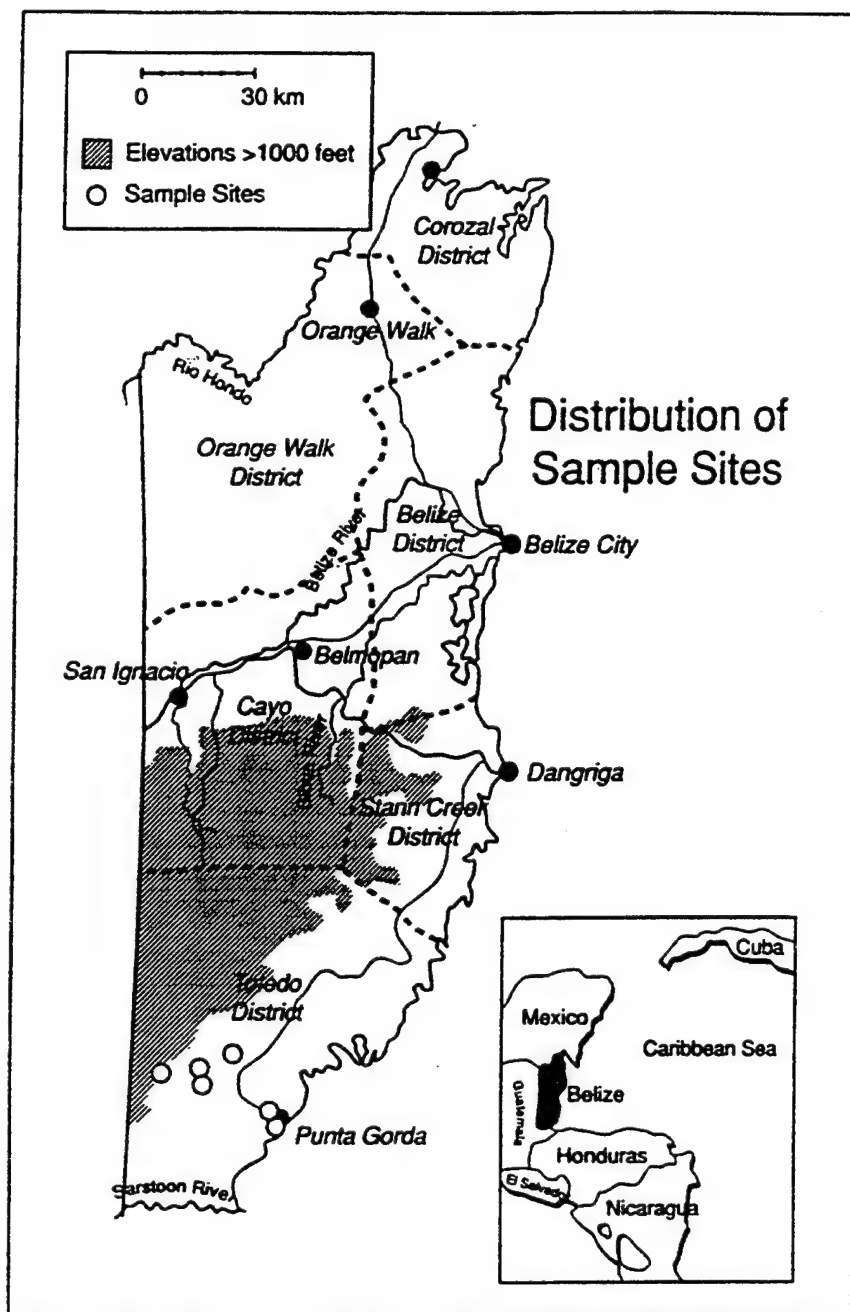


Fig. 1. Map of Belize depicting the distribution of collection sites in Toledo district, near Punta Gorda, Belize.

1900 h) in activity for *An. darlingi* populations in Honduras. Although we employed uniform collecting methods, we could not control for the numbers of children and adults who gathered around the collectors at both indoor and outdoor

collecting sites. Although the collection data were not strongly quantitative, observations on relative composition of indoor versus outdoor collections seemed valid. Most houses had dirt floors and were constructed with loose-fitting wood or

Table 1. Numbers of anophelines collected from humans in paired indoor-outdoor landing collections in the Punta Gorda area of southern Belize during August and September 1992.

Collection site	<i>Anopheles albimanus</i>	<i>Anopheles vestitipennis</i>	<i>Anopheles apicimacula</i>
	Inside/outside	Inside/outside	Inside/outside
Jacinto Landing	2/3	27/4	0/0
Santa Helena	2/1	0/0	0/2
Crique Maffredi ¹	5/2	39/7	7/29
Crique Trosa ¹	0/2	9/0	9/33
Punta Gorda	0/2	2/1	0/0
Big Fall	1/25	0/1	0/0
Totals	10/35	77/13	16/64

¹ Houses not sprayed with DDT.

palm slats and a thatch roof. Two houses in Punta Gorda and Big Fall were solid and tightly enclosed.

Collections were performed at 6 sites in the area of Punta Gorda (Toledo District) in southern Belize (Fig. 1). Although collections were conducted in the same general areas surveyed by Kumm and Ram (1941), due to demographic changes during the intervening 51 years, their specific sites were no longer in existence. In addition to the nighttime landing collections, we conducted larval collections at 145 sites in Toledo District. Specimens from all categories of collections have been deposited in the mosquito collection of the Walter Reed Biosystematics Unit at the Smithsonian Institution.

No larvae or adults of *An. darlingi* were encountered during our survey. The dominant species collected biting indoors was *An. vestitipennis* (Table 1). Both *An. albimanus* and *An. vestitipennis* were widely distributed, being present at 6 and 5 sites, respectively. Overall, larger proportions of *An. albimanus* (78%) and *Anopheles apicimacula* Dyar and Knab (80%) were collected outdoors than indoors. In contrast, 86% of all *An. vestitipennis* females were collected indoors. All 3 *Anopheles* species collected in landing captures were also represented in the larval collections.

Collection data presented herein indicate that *An. darlingi* is either restricted to specific localities that we did not sample, very uncommon, or possibly absent in Toledo District. In April and May 1993, we finally encountered populations of *An. darlingi* adults in riverine environments of Stann Creek District. As a consequence, we think *An. darlingi* is probably present in Toledo District, but is limited to specific riverine localities.

Although *An. vestitipennis* was not encountered in Toledo District during 1940, it was numerically dominant inside houses during our sur-

vey. This species seemed undeterred by DDT residues because large numbers of *An. vestitipennis* females were captured inside both DDT-sprayed and unsprayed houses. Another intriguing aspect of this species' behavior relates to our capturing many more inside houses than were captured outdoors. The openness of many native houses in rural southern Belize probably facilitates this indoor-feeding behavior. In contrast, the host-seeking females of *An. apicimacula*, like *An. albimanus*, were much more abundant outdoors. Exophagic behavior of the latter 2 species should serve to diminish their overall vectorial capacity.

Recent studies by Loyola et al. (1991) and Padilla et al. (1992) have incriminated *An. vestitipennis* as a vector of human malaria in the Marqués de Comillas region of southern Mexico and in 2 communities in northern Guatemala, respectively. The latter studies, in combination with recent data compiled by Padilla in Guatemala (personal communication), show *An. vestitipennis* to be endophagic, but not as strongly endophagic as indicated by our data from Toledo District. Consequently, a greater sampling effort covering the entire nighttime interval will possibly show a greater relative tendency of *An. vestitipennis* females to feed outdoors in Toledo District.

The presence and abundance of malaria vectors are under the control of dynamic environmental variables, as well as human interventions. This report emphasizes the need to continuously study the changing roles of malaria vectors in different geographical areas. Based on the published literature, we can expect *An. darlingi*-transmitted malaria to respond favorably to a DDT house-spray program (Rozendaal et al. 1989, Roberts and Alccrim 1991). However, these expectations must be reevaluated if *An. vestitipennis* has become the primary vector of malaria in nonriverine areas of Toledo District.

Hopefully, this report will be the precursor of more definitive studies on vector responses to DDT-sprayed houses and on vectorial roles in different ecological zones of Belize.

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June 2, 1994

**Seroprevalence of Hepatitis B Virus, Human Immunodeficiency Virus Type-1, and Syphilis Among
Women Attending Prenatal Clinics in Belize, Central America.**

Running Title: Hepatitis B, HIV-1, and Syphilis in Pregnant Women

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Disclaimer: The views expressed herein are those of the authors and do not necessarily represent
those of the Public Health Service or the Department of Defense.

Abstract:

We initiated hepatitis B (HB) screening of women attending prenatal clinics in Belize. Risk factors for HB infection and demographic data were determined by interview. Of 548 women, 81, 14.7%, were seropositive for HB Core Antibody (anti-HBc); one woman had asymptomatic HB surface antigenemia. Antibodies to HIV-1 were detected in one woman. Reactive syphilis serologies were detected in 15 women. Anti-HBc seroprevalence varied by district (range 3.1-43.5%) and by ethnicity (range 0.0%-40.9%). Significant identified risks for anti-HBc seropositivity from univariate analyses included: being of the Garifuna ethnic group (Odds Ratio (OR) 5.4, 95% Confidence Interval (CI) 3.0-10.0), residence in the Stann Creek district (OR 7.7, CI 4.4-13.4), a reactive syphilis serology (OR 5.1, CI 1.4-18.2), a household size of 8 or greater (OR 2.2, CI 1.1-4.3), and five or more lifetime sexual partners (OR 3.2, CI 1.3-8.0). Health care work, tattoos and intravenous drug use were not identified risks. Multivariate analyses identified only ethnicity and a reactive RPR as significant predictors of anti-HBc seropositivity. Strategies to screen pregnant woman and provide immunoprophylaxis to susceptible infants may be effective in interrupting HB transmission in Belize. Highly variable differences in anti-HBc rates by district may permit the targeting of limited public health resources for HB education, screening and prevention programs. Women attending prenatal clinics may be a good population in which to monitor the HIV-1 prevalence in Belize.

Hepatitis B is a common infection with serious sequellae in some developing countries. A previous serologic study of hepatitis virus markers in Belize identified an endemic focus of hepatitis B virus in this Central American country¹. In addition, a study of Belizean Defence Force (BDF) members identified a high overall hepatitis B seroprevalence, 56%, among one ethnic group, the Garifuna². The Garifuna, descendants of African slaves and Carib Indians, still maintain their own culture and language. Two-thirds of Belize's Garifuna population reside in the Stann Creek and Toledo districts. In the BDF study, 11.6% of the Garifuna BDF personnel were seropositive for antibody to hepatitis B core antigen (anti-HBc) and hepatitis B surface antigen (HBsAg). The point prevalence of hepatitis B markers among Garifuna BDF personnel varied little among the age groups tested. This high prevalence of HBsAg with little variation in seroprevalence rates by age suggests perinatal or early childhood hepatitis B transmission or recent adult exposure.

Vertical transmission of hepatitis B virus from mother to infant is an efficient mode of hepatitis B virus infection. Infants born to mothers positive for hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) have a 70-90% chance of acquiring hepatitis B infection perinatally and 85-90% of these infected infants will become chronic hepatitis B carriers.^{3,4} Chronic hepatitis B virus infections are associated with chronic liver disease and hepatocellular carcinoma.⁵

To prevent the perinatal transmission of hepatitis B, infants born to a HBsAg positive mother should be administered hepatitis B immune globulin and the first dose of a three dose series of hepatitis B vaccine within 12 hours of birth. These infants should be followed up to receive the second and third doses of the vaccine at one and six months respectively. This regimen has been shown to prevent 85-95% of perinatal hepatitis B infections.⁶ The Centers for Disease Control and Prevention has recommended screening of all pregnant mothers in the United States for HBsAg and appropriate treatment for identified infants at-risk.⁷

A study was conducted using an anonymous, unlinked, cross-sectional design in order to estimate the current point prevalence of hepatitis B markers among pregnant women attending prenatal clinics in Belize, Central America and to determine risk factors associated with hepatitis B markers in this population. The seroprevalence of antibody to the Human Immunodeficiency Virus (HIV-1) and syphilis serologies were also assessed among this population.

Materials and Methods

This study was an anonymous, unlinked, cross-sectional seroprevalence study of pregnant women attending prenatal clinics affiliated with district hospitals in any of the six districts of Belize over the period July 7, 1992 through August 20, 1993. To contrast seroprevalence rates between ethnic groups, the initial study goals were to target women attending clinics in Stann Creek district and in Orange Walk district, two communities with very

different ethnic components. Once underway, permission was granted by The Ministry of Health in Belize to obtain a more representative sampling of the prenatal population by enrolling women from all six districts in Belize.

Community health nurses routinely draw blood from women attending prenatal clinics for hematocrit, syphilis serologies, and sickle cell preparation. These specimens are tested in a central laboratory in Belize City. Women requiring routine prenatal laboratory examinations were eligible for enrollment into the study. Study specimens were obtained from excess sera submitted for routine prenatal screening.

To obtain a study population that was representative of the overall Belizean population, clinics associated with district hospitals were chosen for study based on: willingness to participate in the study, expected number of prenatal women in attendance at the clinic (larger clinics were preferred), and expected ethnic make-up of women attending the clinic.

At the time of specimen collection, study group members administered a detailed questionnaire to volunteers in either spanish or english, as appropriate. Multi-lingual interpreters at the clinic sites assisted in administering the questionnaire to volunteers speaking other languages. Enrollees were asked: age, ethnicity, place of residence, place of birth, marital status, number of children, previous history of hepatitis or exposure to hepatitis, history of jaundice, history of blood transfusions, history of receiving medicine by injection from a healer, history

of receiving blood-letting remedies from a healer, history of tatoos, age of first intercourse, and number of lifetime sexual partners. A specific history of intravenous drug use was elicited after the first 121 women were interviewed. Blood specimens were linked with questionnaire data at the time of interview by a study number. Only this assigned number linked test results with survey responses. Personal identifiers were not recorded.

Since laboratory testing was performed on sera obtained during routine prenatal screening and results were not linked by identifiers, the study was judged to be exempt from review and the use of an informed consent by the Human Use Committee of the Ministry of Health of Belize and by the Human Use Review Committee at the Uniformed Services University of the Health Sciences.⁸

Laboratory Coordination

Initial serological studies were performed by laboratory technicians at the Epidemiologic Research Center in Belize maintained by the Uniformed Services University of the Health Sciences. Sera were tested in Belize for HBsAg by NML® enzyme-linked immunosorbent assay (ELISA) and for anti-HBc by Hepanostika® both from (Organon Teknika Corporation, Durham NC). All sera reactive for anti-HBc or HBsAg as well as selected samples of non-reactive sera were retested at the Walter Reed Army Institute of Research (WRAIR) by ELISA for anti-HBc by Coryzyme® and HBsAg by Auszyme® (both from Abbott Laboratories, Chicago, Illinois). To further validate anti-HBc seropositivity, 43 of 80 specimens positive for anti-HBc but negative for HBsAg were tested for

hepatitis B surface antibody (anti-HBs) by AUSAB® ELISA at WRAIR.

Sera were tested in Belize for reactivity to rapid plasma reagin (RPR) using the Macro-Vue® RPR 18 millimeter circle card test (Brewer Diagnostic Kits). Sera found to be reactive for RPR in Belize were tested again by ELISA at the National Naval Medical Center in Bethesda, Maryland. In addition, an ELISA test was performed to detect the presence of IgG or IgM antibodies against antigenic treponeme components. Those sera positive for RPR in which IgG was also detected were considered reactive syphilis serologies. Antibodies to the HIV-1 were detected in Belize by Vironostika® ELISA (Organon Teknika Corporation, Durham, NC). Western blot testing of the one positive specimen is pending.

Statistical Analyses

Univariate analyses were performed using two by two tables of a single stratum of a variable cross-tabulated against the other strata taken together. Odds ratios and 95% confidence intervals were calculated by using Epi Info version 5.00 Statcalc.⁹ Significance testing of the differences was accomplished by chi square analysis with Yates' correction or by Fisher's exact test, where applicable. Multivariate analyses of risk factors was done by a stepwise logistic regression model on the Statistical Analysis System (SAS)¹⁰. The usual criteria of alpha equals 0.05 was used for all statistical analyses.

In order to compare the sampled population with the overall pool of pregnant women in a district, the estimated point

prevalence of pregnancy by district was calculated using 1991 district birth rates (number of live births per 1000—district population per year) to approximate the incidence density of pregnancy and 1991 district population estimates in the equation below:¹¹

$$\text{Prevalence} = (\text{Incidence Density}) * (\text{Duration})$$

The duration of pregnant was considered .75 years.

Results:

A total of 548 prenatal women were enrolled. Table 1 compares the estimated prevalence of pregnant women by district to the number of prenatal women enrolled in our study from these districts. Our study population included 11.7% of the country-wide estimated point prevalence of 4692 pregnant women. Prenatal women from Stann Creek and Orange Walk districts were initially targeted for study and are over-represented in our study population. Data on the racial/ethnic composition of pregnant women or women of childbearing age in Belize is unavailable, however, the racial/ethnic composition of the study population in comparison with the racial/ethnic make-up of the overall population of Belize is shown in Table 2. All of the five major ethnic groups are well-represented in our sample.

Adequate sera were available for 543 of 548 enrolled volunteers. As shown in Table 3 eighty-one of 543 specimens (14.7%) tested positive for anti-HBc. Both HBsAg and anti-HBc were detected in only one woman, a 0.1% seroprevalence. Anti-HBs was detected in 35 of 43 (81%) selected specimens with anti-HBc

but no HBsAg. Only one woman (0.1%) was seropositive for antibodies to the Human Immunodeficiency Virus Type 1 (HIV-1). Hepatitis B markers were not detected in her sera, and her syphilis serology was non-reactive. Fifteen women (2.7%) had a reactive RPR; only two of these did not have IgG anti-T. pallidum by ELISA, each with an RPR titer of only 1:1.

Table 4 shows anti-HBc seroprevalence rates by demographic factors and sexual practices. Single women were significantly more likely than married, common-law, divorced, or separated women to be anti-HBc seropositive, and women in the 30 to 34 year old age group were significantly more likely to be anti-HBc seropositive than women in other age groups. Serologic evidence of exposure to hepatitis B virus was significantly associated with a household size greater than eight, and a history of five or more lifetime sexual partners. Women of the Garifuna ethnic group, and women born or residing in Stann Creek were significantly more likely to be anti-HBc seropositive than women from other ethnic groups or women born or living in other districts. The lowest anti-HBc seroprevalence rates were among German/Dutch mennonite women in the Cayo district, 0 of 19 (0%).

Table 5 depicts epidemiologic risk factors for hepatitis B infection. Reactive syphilis serologies were significantly associated with hepatitis B markers. Other known risk factors for hepatitis B viral infection were not found to be associated with evidence of hepatitis B markers. Although a relatively large component of women had histories of transfusion, 8.5%, and

tattoos, 7.7%, these factors were not associated with hepatitis B markers among our study population. —

Multivariate Logistic Regression:

To pursue the individual importance of potentially correlated factors in determining the seroprevalence of hepatitis B markers, we performed multivariate analyses by stepwise logistic regression. All the variables that were significantly associated with hepatitis B exposure in the univariate analyses were included in the multivariate model. Using the usual inclusion/exclusion criteria of 0.05, we found that ethnicity and a reactive RPR were the only significant independent predictors of anti-HBc seroprevalence.

Discussion:

Univariate analyses identified many factors that might significantly contribute to finding serologic evidence of hepatitis B infection in our population. The predominant mode of hepatitis B transmission in our study population is unclear. Heterosexual transmission of hepatitis B infection is suggested by anti-HBc association with multiple sexual partners, and reactive syphilis serologies. This finding is consistent with several recent studies which underscore the importance of the sexual mode of hepatitis B infection in persons with adult acquired disease.^{12,13,14,15} Hepatitis B virus acquisition by a horizontal mode of transmission is suggested by high anti-HBc seroprevalence rates identified with increasing household size. In this study, many of the parenteral risk factors for

transmission of hepatitis B virus (intravenous drug use, history of blood transfusions, or history of tattoos, etc.) were not found to be significantly associated with serologic evidence of infection. Multivariate analyses found ethnicity and a reactive RPR to be the only significant independent predictors of hepatitis B markers.

Our study supports the findings of a previous study in Belize conducted among members of the Belizean Defence Force (BDF).² The BDF study found serologic evidence of hepatitis B infection to be highest in the Garifuna ethnic group. High rates of hepatitis B virus have been observed in Africans living in sub-Saharan Africa¹⁶ and among the Carib Indians of South America¹⁷, the two ethnic groups from which the Garifuna descended. Differences in seroprevalence rates by ethnicity may be due to socioeconomic, cultural, or other factors rather than ethnicity directly. Further studies are needed to define the predominant mode of hepatitis B transmission among the Garifuna.

In the BDF study of predominantly male subjects², the seroprevalence of both HBsAg and anti-HBc was found to be 4% overall and 11.6% among the Garifuna. In contrast, only one woman (a Garifuna) in our study was seropositive for both HBsAg and anti-HBc, a 0.1% seroprevalence rate. The high rate of anti-HBs in those with anti-HBc confirms clearance of HBsAg after hepatitis B virus infection. The reason for the apparent disparity between the hepatitis B surface antigen seroprevalence rates among the women enrolled in the present study and the

predominantly male study population of the BDF study is unclear. Females are more likely to clear the surface antigen than males. In a study to determine childhood hepatitis B infection rates in two Gambian villages in West Africa, male children infected with hepatitis B virus were significantly more likely to be carriers of HBsAg and hepatitis B e antigen than were female children.¹⁸ This higher rate of HBsAg in males has been postulated as one reason for the male predominance of hepatocellular carcinoma; more than 80% of the cases of hepatocellular carcinoma occur in males.^{16,19}

Epidemiologic risk factors for hepatitis B due to parenteral or blood exposure did not significantly contribute to the high hepatitis B seroprevalence in the present study. Blood transfusion, while reported by 8.4% of the the women, was not a significant risk factor. This potential source of hepatitis B transmission has been interrupted in Belize by the routine screening of blood donors for HBsAg. However, other parenteral modes of transmission might have contributed to high seroprevalence rates. Needles and syringes were sterilized and re-used until the early 1980s when disposable needles and syringes became available. In addition, the possibility of parenterally transmitted hepatitis B virus from dental procedures, a source of occasional outbreaks, was not addressed in this study.^{20,21}

Criteria set forth by the Expanded Programme on Immunization (EPI), a multinational effort to immunize all of the world's

children against the immunizable diseases of childhood, recommends universal hepatitis B immunization of infants in countries in which the hepatitis B virus carrier rate is 2.5%.²²

Based on these criteria, this study does not identify a need for universal vaccine coverage for all infants born in Belize.

However, the rate of HBsAg of ,1.5%, among pregnant Garifuna women and the 11.6% rate among predominantly male Garifuna soldiers suggests that vaccination of infants in Stann Creek district may be of benefit. All infants in Stann Creek district should be targeted for this vaccination campaign, since a previous study determined a high prevalence of hepatitis B serologic markers among another population living in Stann Creek district, migrant farmworkers living on banana plantations.¹

These farmworkers were not sampled in the present study.

Furthermore, Stann Creek district has the highest reported incidence of hepatitis cases in Belize (Ministry of Health, Belize).

While the Stann Creek district has the highest hepatitis B seroprevalence rates and would benefit from the universal administration of hepatitis B vaccine to newborns, the overall seroprevalence rate of hepatitis B markers found in this study, 15%, is about three times that of women of childbearing age (ages 14-40) in the US.²³ A comprehensive strategy to screen pregnant women for hepatitis B and provide active/passive immunization for susceptible newborns born in districts other than Stann Creek is reasonably recommended from our findings. The significant

variability in seroprevalence rates by district permits the targeting of precious public health resources to districts most in need of hepatitis B education, screening, and immunization programs.

HIV-1 antibodies were detected in only one woman. No extrapolation of an overall seroprevalence for Belizean women can be made from this one observation. However, the rapid expansion of heterosexually transmitted HIV-1 infection in women in the United States²⁴ suggests the potential for spread in women in Belize as well.²⁵ Furthermore, the recent finding that administration of AZT to pregnant women significantly decreases transmission of HIV-1 to their children gives greater importance to screening pregnant women for HIV-1 and other sexually transmitted diseases.²⁶ Another value in testing for HIV-1 in pregnant women in Belize would be to monitor the prevalence of HIV-1 in the general population since much of the transmission of HIV-1 in Belize would be expected to be heterosexually.

That syphilis, hepatitis B, and other sexually transmitted diseases are associated with HIV-1 infection emphasizes the need to prevent and treat all sexually transmitted diseases.²⁷ Women (and men) presenting with a sexually transmitted disease should be educated about hepatitis B and HIV-1 and offered counseling, testing and hepatitis B vaccine, as available. Control of sexually transmitted diseases will decrease the potential for transmission of HIV-1 in Belize in the future. The addition of testing for HIV-1 and HBsAg to existing prenatal tests may be

useful in the control of these and other sexually transmitted diseases as well as identifying infants at risk for neonatal hepatitis B.

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Table 1

Comparison of Estimated Prevalence of Prenatal Women and Actual Study
Enrollment, by District

District	Estimated No. of Prenatal Women	Actual Enrollment No. (% of Est.)
Belize	1662	143 (8.6%)
Cayo	892	68 (7.6%)
Corozal	582	65 (11.2%)
Orange Walk	707	135 (19.1%)
Stann Creek	387	93 (24.0%)
Toledo	462	44 (9.5%)
All	4692	548 (11.7%)

Table 2

Comparison of the Proportion of Ethnic Groups in the Population of Belize and Proportion of Ethnic Groups in the Actual Study Enrollment

Ethnic Group	% of Population*	Actual
		Enrollment
		No. (% of Enrollment)
Creole	29.8%	125 (22.8%)
Garifuna	6.6%	66 (12.0%)
Mestizo/Spanish	43.6%	258 (47.1%)
Mayan	5.0%	32 (5.8%)
East Indian	3.5%	34 (6.2%)
Dutch-German/Mennonite	3.1%	19 (1.4%)
Other/Unknown	5.1%	19 (3.5%)
Total	100%	548 (100%)

* Bases on 1991 Census

Table 3
Summary of Seroprevalence Findings

	No. Tested	%
Anti-HBc (+)	81/543	14.7%
HBsAg (+)	1/542	0.1%
Anti-HBs (+)*	35/43	81.0%
Reactive RPR**	13/542	2.4%
Anti-HIV-1	1/543	.2%

* 43 of 80 specimens with anti-HBc alone were tested for anti-HBs

** Confirmed by ELISA

Anti-HBc = Anti-hepatitis B core antigen

HBsAg = Hepatitis B surface antigen

Anti-HBs = Anti-hepatitis B surface antigen

RPR = Rapid Plasma Reagin

Table 4
Characteristics of Prenatal Belizean Women by Presence of Anti-HBc, Belize 1993

	No.	Seroprevalence Rate (%)	OR	95%CI	p-value (if significant)
MARITAL STATUS					
Single	20/89	22.5%	1.9	1.0, 3.4	p=.04
Married	31/218	14.2%	.9	.6, 1.5	
Common-law	29/223	13.0%	.6	.4, 1.0	
Separated	0/7	0.0%			
Unknown	1/6	16.7%			
AGE (mean=24.1, SD=6.1)					
14-19	20/133	15.0%	1.1	.6, 1.9	p=.04
20-24	23/176	13.1%	.9	.5, 1.5	
25-29	13/120	10.8%	.7	.3, 1.3	
30-34	16/68	23.5%	2.0	1.0, 3.9	
35-39	4/30	13.3%	.9	.3, 2.9	
>40	1/7	14.3%	1.0	0.0, 8.3	
Unknown	4/9	44.4%			
CHILDREN (mean=2.1, SD=2.5)					
0	28/172	16.3%	1.2	.7, 2.0	
1-4	38/302	12.6%	.7	.4, 1.1	
>5	15/69	21.7%	1.7	.9, 3.3	
HOUSEHOLD SIZE (mean=5.1, SD=2.6)					
1-4	39/276	14.1%	.9	.5, 1.5	p=.02
5-8	26/204	12.7%	.8	.4, 1.3	
>8	16/62	25.8%	2.2	1.1, 4.3	
Unknown	0/1	0.0%			
NO. PARTNERS (mean=1.5, SD=1.9)					
1	37/315	11.7%	.6	.3, .8	p=.003
2	19/103	18.4%	1.5	.8, 2.7	
3-4	13/93	14.0%	.9	.5, 1.8	p=.01
>5	9/25	36.0%	3.2	1.3, 8.0	
Unknown	3/7	42.9%			
AGE AT FIRST SEX (mean=17.2, SD=2.8)					
10-14	11/67	16.4%	1.2	.5, 2.4	
15-19	60/390	15.3%	1.3	.7, 2.4	
>20	7/80	8.8%	.5	.2, 1.2	
Unknown	3/5	60.0%			
ETHNICITY					
Creole	24/125	19.2%	1.4	.8, 2.5	p<.000000001
Garifuna	27/66	40.9%	5.4	3.0, 10.0	
Mayan	7/32	21.9%	1.7	.6, 4.2	p=.000006
Mestizo/Spanish	21/258	8.1%	.3	.2, .6	
East Indian	2/34	5.9%	.3	.2, 1.5	
Other/Unknown	0/27	0.0%			
DISTRICT					
Orange Walk	8/143	5.6%	.3	.1, .6	p=.0007
Stann Creek	40/92	43.5%	7.7	4.4, 13.4	p<.000000001
Belize	16/132	12.1%	.7	.4, 1.4	
Toledo	11/44	25.0%	2.0	.9, 4.4	
Cayo	4/68	5.9%	.3	.1, .9	
Corozal	2/64	3.1%	.2	0.0, .6	p=.04
PLACE OF BIRTH					
Orange Walk	3/77	3.9%	.2	0.0, .6	p=.004
Stann Creek	35/64	54.9%	18.7	10.1, 34.7	p<.000000001
Belize	16/153	10.5%	.6	.3, 1.1	p=.05
Toledo	8/45	17.8%	1.3	.5, 3.0	
Cayo	2/45	4.4%	.3	0.0, 1.0	
Corozal	1/30	3.3%	.2	0.1, 1.1	
Neighboring Countries	15/103	14.6%	.9	.5, 1.7	
Other/Unknown	1/24	4.2%			

Table 5

Epidemiologic Factors by Presence of Anti-HBc, Belize 1993

	No.	Rate* (%)	OR**	95%CI***	
EPIDEMIOLOGIC FACTORS					
Transfusion	7/46	15.2%	1.0	.4, 2.5	NS****
HCW*****	2/8	25.0%	2.0	.2, 11.2	NS
Injections	2/5	23.8%	1.8	.6, 5.5	NS
Tattoo	5/40	12.5%	4.0	.5, 30.0	NS
STD Hx	3/14	9.0%	1.6	.4, 6.3	NS
IVDU	1/3	33.3%	2.8	0.0, 41.0	NS
RPR (+)	6/13	46.2%	5.1	1.4, 18.2	p=.02
Hepatitis Hx	2/22	9.0%	.6	.1, 2.6	NS

* Seroprevalence rate

** Odds Ratio relative to those without this potential risk

*** 95% Confidence Interval

**** Not significant

***** Health Care Worker

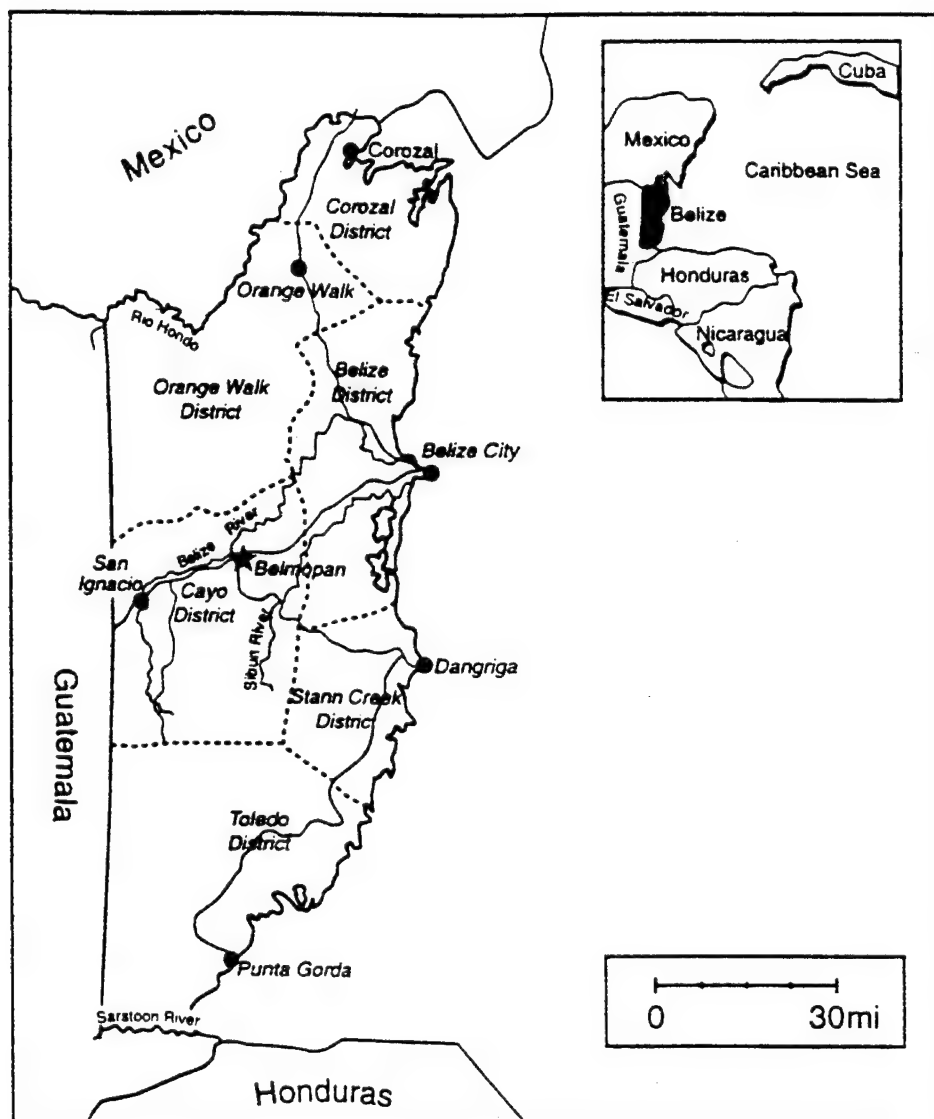


Figure 1: Map of Belize, Central America indicating the 6 districts and their capitals.

Characterization of a prototype strain of hepatitis E virus

(anti-hepatitis E virus antibodies/PCR/genome sequence comparison)

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Contributed by Robert H. Purcell, October 10, 1991

ABSTRACT A strain of hepatitis E virus (SAR-55) implicated in an epidemic of enterically transmitted non-A, non-B hepatitis, now called hepatitis E, was characterized extensively. Six cynomolgus monkeys (*Macaca fascicularis*) were infected with a strain of hepatitis E virus from Pakistan. Reverse transcription-polymerase chain reaction was used to determine the pattern of virus shedding in feces, bile, and serum relative to hepatitis and induction of specific antibodies. Virtually the entire genome of SAR-55 (7195 nucleotides) was sequenced. Comparison of the sequence of SAR-55 with that of a Burmese strain revealed a high level of homology except for one region encoding 100 amino acids of a putative nonstructural polypeptide. Identification of this region as hypervariable was obtained by partial sequencing of a third isolate of hepatitis E virus from Kirgizia.

Epidemics of enterically transmitted non-A, non-B hepatitis (hepatitis E) have been reported in Asia, Africa, and North America (for reviews, see refs. 1-4). Similar cases of sporadic hepatitis, presumed to be hepatitis E, account for up to 90% of reported hepatitis in countries where hepatitis E is endemic. Furthermore, hepatitis E has been implicated in fulminant hepatitis of pregnancy: up to 20% of pregnant women with hepatitis E die of their disease. That a viral agent was responsible for hepatitis E epidemics was first shown by Balayan *et al.* (5). It was provisionally called hepatitis E virus (HEV) (2). On the basis of electron microscopy and other characterization it was proposed that HEV belongs to the calicivirus family (6, 7). A partial sequence (8) and later the entire sequence (9) of an HEV genome have been reported. Comparisons of HEV sequences with sequences available in computerized data bases, including those of caliciviruses (10), showed little similarity between HEV and any other known viruses.

Several species of primates have been experimentally infected with HEV (5, 11-19). Although HEV infection is less severe in monkeys than in humans, it is sufficiently similar that monkeys can serve as a reasonable and reproducible animal model to provide a more complete characterization of this infection.

Determination of the pattern of HEV shedding in feces, bile, and sera during infection is important for characterization of the disease and elucidation of the epidemiology of HEV. In some cases, the presence of HEV in a sample has been demonstrated by transmission to animals (5, 11-19) but such transmission studies cannot be used for routine investigation. Until recently, the primary method available for characterization of virus shedding was immune electron microscopy (IEM) (for reviews, see refs. 1-4). However,

because the level of HEV in feces and bile is very low (5, 11-19), the sensitivity of IEM is inadequate for complete characterization of HEV infection. A more sensitive technique, detection of the virus genome by reverse transcription-polymerase chain reaction (RT-PCR), was used in this study to correlate the presence of HEV in serum, bile, and feces of an experimentally infected cynomolgus monkey with biochemical evidence of hepatitis and development of antibodies to HEV (anti-HEV). In addition, we obtained structural information about the virus genome through sequencing of the PCR products themselves or their cloned derivatives.**

MATERIALS AND METHODS

Virus Samples and Inoculation of Primates. Feces containing HEV SAR-55 were collected from a patient during a hepatitis E outbreak in Sargodha, Pakistan (20, 21). Approximately 0.5 ml per monkey of a 10% (wt/vol) stool suspension of feces in fetal calf serum was used for intravenous inoculation of six cynomolgus monkeys (*Macaca fascicularis*). Blood samples from these monkeys were taken approximately twice weekly before and after inoculation and tested by Metpath (Rockville, MD) for biochemical evidence of hepatitis by measuring levels of serum alanine aminotransferase, isocitric dehydrogenase, and γ -glutamyltransferase. Fecal and bile samples were also collected from one monkey. For daily collection of bile, implantation surgery was performed on the 7th day after inoculation to establish an indwelling T-tube into the bile duct.

Bile from a cynomolgus monkey infected with another strain of HEV (OSH-1852) was kindly provided by Michael Balayan (Institute of Poliomyelitis and Viral Encephalitis, Moscow). This cynomolgus monkey had been inoculated with feces collected during a hepatitis E outbreak in 1988 in Osh, Kirgizia, U.S.S.R. Bile was collected on the 14th day after infection.

Detection of Anti-HEV Antibodies. A modified ELISA protocol (22) was used in this study. Recombinant HEV antigens for use in the ELISA were derived from Mexican and Burmese strains (23, 24) and were produced in the pGEX1 vector system (25). Cynomolgus sera were diluted 1:100 in 1% gelatin/phosphate-buffered saline (PBS). Alkaline phosphatase-conjugated goat anti-human IgG was used as a second antibody.

Abbreviations: HEV, hepatitis E virus; IEM, immune electron microscopy; RT, reverse transcription; nt, nucleotide(s); ORF, open reading frame.

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**The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M80581 and M81415).

Primers. Ninety-two primers, 21–40 nucleotides (nt) long, and complementary to plus or minus strands of the genome of a strain of HEV from Burma (BUR-121) (9) or the SAR-55 genome were synthesized using an Applied Biosystems model 391 DNA synthesizer. For cloning of PCR fragments, *EcoRI*, *BamHI*, or *Bgl II* restriction sites preceded by 3–7 nt were added to the 5' end of primers.

For detection of the HEV genome in samples of bile, sera, and feces, two sets of "nested" primers were used that represented sequences from the 3' region (ORF-2) of the SAR-55 genome. Primers for RT and the first PCR were 5'-GTATAACGGATCCACATCTCCCTTACCTC-3' and 5'-TACAGATCTATAACAACCTTAACAGTCGG-3' and for the second PCR were 5'-GCGGCAGATCTCACCAGACAC-CATTAGTAC-3' and 5'-TAACCTGGATCCTTATGCCGC-CCCTCTTAG-3'.

Preparation of Virus RNA Template for PCR. Bile (10 μ l), 20% (wt/vol) SDS (to a final concentration of 1%), proteinase K (10 mg/ml; to a final concentration of 1 mg/ml), 1 μ l of tRNA (10 mg/ml), and 3 μ l of 0.5 M EDTA were mixed in a final volume of 250 μ l and incubated for 30 min at 55°C. Total nucleic acids were extracted from bile twice with phenol/chloroform, 1:1 (vol/vol), at 65°C and once with chloroform, then precipitated by ethanol, washed with 95% ethanol, and used for RT-PCR. RT-PCR amplification of HEV RNA from feces and especially from sera was more efficient when RNA was more extensively purified. Serum (100 μ l) or a 10% fecal suspension (200 μ l) was treated as above with proteinase K. After a 30-min incubation, 300 μ l of CHAOS buffer (4.2 M guanidine thiocyanate/0.5 M *N*-lauroylsarcosine/0.025 M Tris-HCl, pH 8.0) was added. Nucleic acids were extracted twice with phenol/chloroform at 65°C followed by chloroform extraction at room temperature. Then 7.5 M ammonium acetate (225 μ l) was added to the upper phase and nucleic acids were precipitated with 0.68 ml of 2-propanol. The pellet was dissolved in 300 μ l of CHAOS buffer and 100 μ l of water was added. Chloroform extraction and 2-propanol precipitation were repeated. Nucleic acids were dissolved in water, precipitated with ethanol, washed with 95% ethanol, and used for RT-PCR.

RT-PCR. The usual 100- μ l RT-PCR mixture contained template, 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM

MgCl₂, all four dNTPs (each at 0.2 mM), 50 pmol of direct primer, 50 pmol of reverse primer, 40 units of RNasin (Promega), 16 units of avian myeloblastosis virus reverse transcriptase (Promega), 4 units of AmpliTaq (Cetus), under 100 μ l of light mineral oil. The mixture was incubated 1 h at 42°C and then amplified by 35 PCR cycles: 1 min at 94°C, 1 min at 45°C, and 1 min at 72°C. The PCR products were analyzed on 1% agarose gels.

Cloning of PCR Fragments. PCR fragments containing restriction sites at the ends were digested with *EcoRI* and *BamHI* or *EcoRI* and *Bgl II* restriction enzymes and cloned in *EcoRI/BamHI*-digested pBR322 or pGEM-3Z (Promega). Alternatively, PCR fragments were cloned into pCR1000 using the TA cloning kit (Invitrogen, San Diego).

Sequencing of PCR Fragments and Plasmids. PCR fragments were excised from 1% agarose gels and purified by GeneClean (Bio 101, La Jolla, CA). Double-stranded PCR fragments were sequenced by using Sequenase (United States Biochemical) as described (26). Double-stranded plasmids purified through CsCl gradients were sequenced with a Sequenase kit (United States Biochemical).

Computer Analysis of Sequences. Nucleotide sequences of HEV strains were compared using the Genetics Computer Group (Madison, WI) software package (27), version 7.5, on a VAX 8650 computer (at the National Cancer Institute, Frederick, MD). This software package was also used to generate the hydropathy plots.

RESULTS

Biological, Serological, and Virological Characterization of Infection. Six cynomolgus monkeys were intravenously inoculated with a 10% suspension of human feces containing the SAR-55 strain of HEV. Biochemical and serological assays confirmed that all six inoculated animals were infected. Although data are shown for only one monkey (Fig. 1), all six monkeys displayed an increase in alanine aminotransferase, isocitric dehydrogenase, and γ -glutamyltransferase activity, indicative of acute hepatitis. All six animals also developed antibody to recombinant-derived HEV antigens. These data demonstrated that the SAR-55 strain of

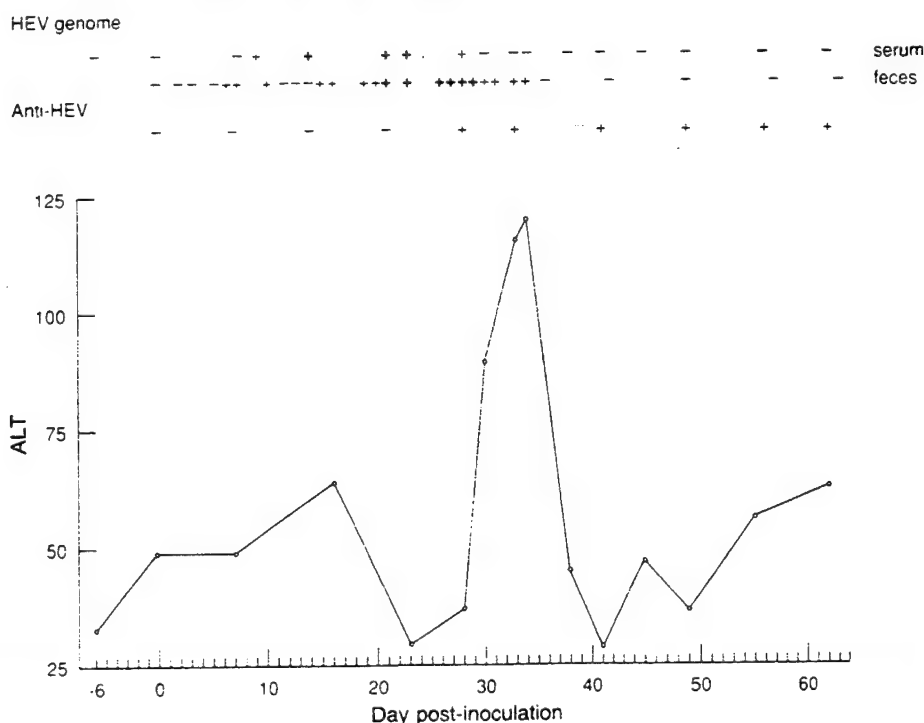


FIG. 1. Evidence for SAR-55 infection of Cyno-376. Serum alanine aminotransferase (ALT) levels are plotted as units/liter and the presence (+) or absence (-) of viral genomes or antibody to HEV is indicated. Viral genomes in the feces and serum were detected by RT-PCR. The presence of total serum antibodies to HEV was monitored by ELISA using recombinant antigens from both the Mexican and Burmese strains of HEV (23, 24).

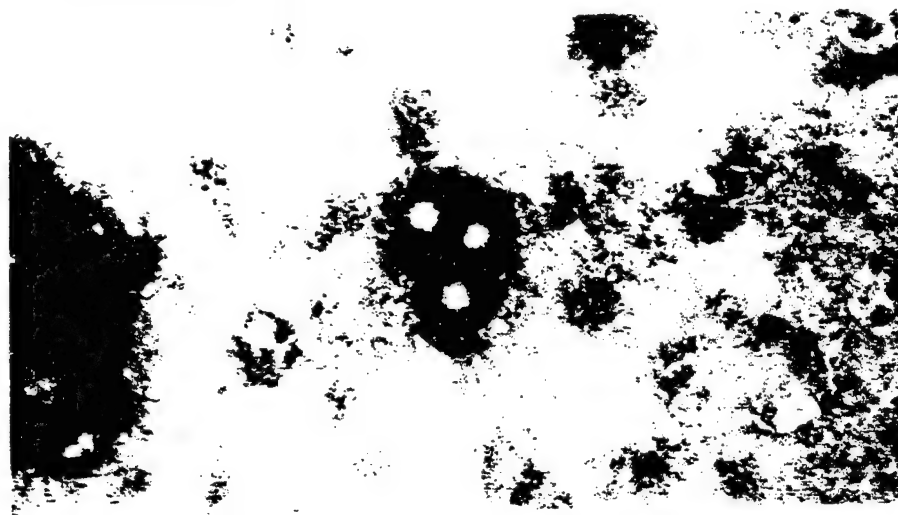


FIG. 2. IEM detection of SAR-55 virions in the bile of Cyno-376. IEM was performed as described (13). Viral aggregates were identified in the bile by reaction of diluted bile with anti-HEV from a chimpanzee that had been infected with a strain of HEV from India (13). ($\times 150,000$.)

HEV was able to consistently infect and induce hepatitis E in cynomolgus monkeys.

To correlate the distribution of virus with an increase in virus-specific antibody and alanine aminotransferase levels, a sensitive nested RT-PCR protocol was performed on fecal, serum, and bile samples from cynomolgus monkey 376 (Cyno-376). Control experiments in which we assayed dilutions of feces of known infectivity demonstrated that on average the HEV genome could be detected by RT-PCR in feces, with a sensitivity approximately equal to that of an assay based on transmission of HEV to cynomolgus monkeys (unpublished results). All bile, serum, and fecal samples were tested by RT-PCR multiple times to ensure that positive samples were identified.

Although Cyno-376 was inoculated with at least 1×10^3 infectious doses intravenously, we were not able to detect virus in serum on the day of infection or for several days thereafter (Fig. 1). However, we were able to detect viral genomes in feces and bile as early as day 6 and day 7, respectively, confirming that excretion of virus was an early indicator of infection. All samples of bile collected (days 7–41) were positive for HEV RNA by RT-PCR. Unfortunately, due to the indwelling catheter method of bile collection, residual contamination from earlier samples could not be eliminated so the end point for virus presence in the bile could not be determined by PCR. However, when the less-sensitive IEM technique was used in the analysis of the bile, virus was detected sporadically from day 26 to day 37 with a peak on day 32 (data not shown). The HEV particles resembled those described previously (Fig. 2).

Viral excretion in feces could be documented more precisely by PCR, and excretion began on day 6 and ended by day 35 (Fig. 1). Three samples, taken between days 10 and 15, were repeatedly negative for viral RNA. The inability to detect viral RNA in these three samples suggested that the level of excreted particles was low. Virus was first detected in the serum on day 9, appeared to be present at higher titer (based on the quantity of PCR product) from days 14 to 23, then disappeared by day 28 (Fig. 1). A similar pattern of viremia in a cynomolgus monkey infected with HEV was observed by Uchida *et al.* (28).

Molecular Characterization of the SAR-55 Genome. RT-PCR amplification of bile from Cyno-376 was employed to generate six cDNA fragments encompassing the entire genome of SAR-55. The PCR-generated fragments were either sequenced directly or were cloned into individual plasmids and sequenced subsequent to amplification in *Escherichia coli*. Direct sequencing of the PCR product provided 45% of the genome sequence as a consensus sequence. Both strands of cloned cDNAs representing 87% of the genome were

sequenced to provide the remainder of the sequence and to confirm the consensus sequence. The sequence of the entire 7.195-kilobase genome, with the exception of 30 and 27 nt of the 5' and 3' termini, respectively, was obtained.

Since one other isolate of HEV, BUR-121 (9), had been totally sequenced, computer analyses were performed to determine the relatedness of SAR-55 to BUR-121. Unique nucleotide insertions or deletions were not detected and the same three ORFs identified in BUR-121 were found in SAR-55. Overall, the genomes were quite similar and differed by only 6.7% in nucleotide sequence and by 1% in deduced amino acid sequence (Table 1). As might be expected, the most conserved region was located in the overlap of ORF-2 and ORF-3 (bases 5147–5477). Against this overall pattern of sequence relatedness, the region between bases 2011 and 2325 in ORF-1 appeared unique (Fig. 3). Although the sum of the nucleotide differences in this region was only 2% higher than the average for the entire genome, the sum of amino acid differences was 14% or 14 times higher than the average. Because the sequence of this region in the SAR-55 genome differed so greatly from that of BUR-121, it was also amplified by RT-PCR directly from the original human feces. The sequence obtained from virus in the human fecal sample was identical to that obtained from virus in the cynomolgus bile.

Identification of a Hypervariable Region. To determine if this extreme divergence of sequence between nt 2011 and 2325 was unique to these two isolates or had a more universal significance, sequences from two regions of a third strain of HEV were derived. Fragments of cDNA from the RT-PCR amplification of the OSH-1852 strain of HEV corresponding to positions 2002–2424 and 4424–4800 of the genome were sequenced. Both the nucleotide and amino acid identities in the region (positions 4424–4800) encoding the putative RNA-directed RNA polymerase were high and did not differ substantially among the three strains (Table 2 and Fig. 3). In contrast, between nt 2002 and 2424 there was significant divergence in nucleotide sequences and even greater divergence in amino acid sequences among the three strains.

Table 1. Comparison of BUR-121 and SAR-55 sequences

Region	Nucleotides		Amino acids	
	Length, no.	Identity, %	Length, no.	Identity, %
ORF-1	5079	93.1	1693	98.8
ORF-2	1980	93.8	660	99.4
ORF-3	369	98.9	123	100
Total	7138*	93.3	2476	99.0

*Length of the primers is not included.

Regions compared are nt 2002–2424 (predicted nonstructural proteins) and nt 4423–4878 (predicted RNA-directed RNA polymerase).

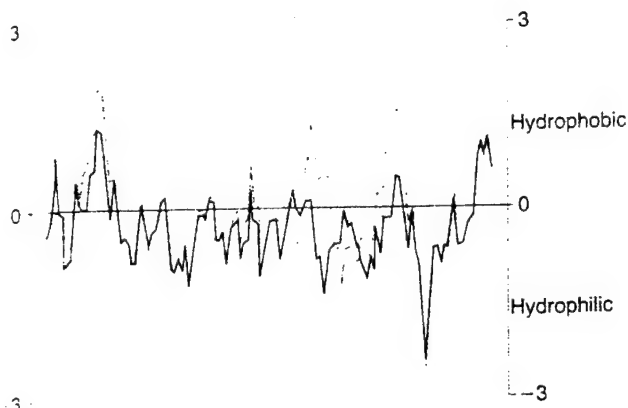


FIG. 4. Hydropathy plot of the hypervariable region shown in Fig. 3 was plotted for each strain using the method of Kyte and Doolittle (30) and profiles were superimposed for comparison. SAR-55, bold line; BUR-121, dotted line; OSH-1852, dashed line.

the day of infection or thereafter until 2–3 days after virus was first detected in bile and feces. Virus excretion started by 1 week after infection and 3 weeks before evidence of disease was observed. The amount of virus excreted at this time was not great, which probably explains why we were not able to detect the HEV genome in fecal samples taken 12–14 days after infection even though samples taken prior to and after this time were positive. Viremia was first observed on the 9th day of the infection and appeared to intensify between 2 and 3 weeks after infection. Elevation of liver enzymes was first noted as viremia ceased. It is probable that viremia is the result of viral replication in the liver although nonhepatic sites of replication have not been ruled out.

Comparison of SAR-55 Genome with That of Other Strains.

We found that the sequence of SAR-55 was quite similar to that of BUR-121 over the entire length of the genome (Table 1). This result is not unexpected since the two viruses were isolated from geographically contiguous regions at about the same time. Differences between the two sequences were distributed approximately uniformly throughout the genome except for one hypervariable domain located in the putative nonstructural ORF (9) (Fig. 3 and Table 2). This region of hypervariability preceded the nucleotide triphosphate binding domain and also the RNA-directed RNA polymerase motifs recognized in ORF-1 (8, 9, 29). That this region was hypervariable was confirmed when another strain from Asia was partly sequenced. The genome of the OSH-1852 strain from Kirgizia differed in the hypervariable region to approximately the same extent from the Pakistani and Burmese strains as those strains differed from each other (Table 2). However, all three strains did not differ significantly from each other in the region coding for RNA-directed RNA polymerase. The finding of a hypervariable region in a presumed nonstructural protein is surprising, since such regions are usually found in structural proteins of viruses, where they are exposed to selection by the immune response of the host. A hydropathy analysis of the region revealed that all three strains displayed a similar profile in spite of their extensive sequence differences (Fig. 4). However, the significance of this pattern remains to be determined. We predict that the existence of this hypervariable region will allow even closely related strains to be distinguished by the application of molecular epidemiological approaches. Presumably, the sequence of the hypervariable region could be used for more detailed molecular classification of HEV strains, and the sequence from the much more conserved

RNA polymerase region could be used for designing PCR primers to detect a spectrum of HEV strains with different origins.

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Infectivity Titration of a Prototype Strain of Hepatitis E Virus in Cynomolgus Monkeys

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The infectivity titer of a standard stock of the SAR-55 strain of hepatitis E virus (HEV) was determined in cynomolgus macaques (*Macaca fascicularis*) and the effect of dose on the course of the infection was examined by weekly monitoring of alanine aminotransferase (ALT) and anti-HEV levels. Antibody to HEV (anti-HEV) was measured with ELISAs based on ORF-2 recombinant antigens consisting of either a 55 kDa region expressed in insect cells or shorter regions expressed as fusion proteins in bacteria. The ELISA based on the 55 kDa antigen was generally more sensitive. The infectivity titer of SAR-55 was 10^6 cynomolgus 50% infectious doses per gram of feces. The infectivity titer corresponded to the HEV genome titer of the inoculum as determined by reverse transcriptase-polymerase chain reaction (RT-PCR). Anti-HEV IgM was detected in only a portion of the animals that had an anti-HEV IgG response. Biochemical evidence of hepatitis was most prominent in animals that were inoculated with the higher concentrations of virus and the incubation period to seroconversion was prolonged in animals that received the lower doses.

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KEY WORDS: HEV genome, PCR, baculovirus expressed antigen, bacteria expressed antigen, anti-HEV

INTRODUCTION

Hepatitis E is a major cause of hepatitis in developing countries [reviewed in Balayan, 1987; Purcell and Ticehurst, 1988; Bradley, 1990; Ticehurst, 1991; Krawczynski, 1993]. Although hepatitis E virus (HEV) was identified as the cause of this disease by Balayan et al. in 1983, the development of practical serological tests for hepatitis E became possible only recently after molecular cloning of the HEV genome [Reyes et al., 1990; Tam et al., 1991; Tsarev et al., 1992; Huang et al., 1993] and expression of viral proteins [Yarbough et al.,

1991; Dawson et al., 1992; Favorov et al., 1992; Tsarev et al., 1993b]. The reverse transcriptase-polymerase chain reaction (RT-PCR) technique made it possible to detect HEV genomes in experimental and clinical specimens [Uchida et al., 1991; Tsarev et al., 1992; Chauhan et al., 1993], while the development of serologic tests permitted the detection of anti-HEV in serum [Yarbough et al., 1991; Dawson et al., 1992; Tsarev et al., 1992, 1993a,b]. Thus extensive experimental, clinical and epidemiological studies of HEV are now possible. Experimental studies can best be evaluated when a well characterized virus inoculum is used. For this reason we have used both PCR and immunological techniques to estimate the infectious titer of the SAR-55 strain of HEV in cynomolgus monkeys relative to the virus genome titer of the inocula. In addition, three different recombinant antigens expressed from open reading frame 2 (ORF-2) of the virus in bacteria or insect cells [Yarbough et al., 1991; Tsarev et al., 1993b] were compared for their efficiency in detecting and quantifying anti-HEV during the course of infection.

MATERIALS AND METHODS

Virus Samples and Inoculation of Primates

Feces containing the SAR-55 strain of HEV were collected from a patient during a hepatitis E outbreak in Sargodha, Pakistan in 1987 [Iqbal et al., 1989; Ticehurst et al., 1992a, Bryan et al., 1993]. A 10% stool suspension of the feces in fetal calf serum was serially diluted ten-fold in steps, aliquoted, and stored in liquid nitrogen. One half ml of the diluted fecal sample was used for intravenous (10^{-1} – 10^{-8}) or oral (10^{-1}) inoculation of 18 cynomolgus monkeys (*Macaca fascicularis*). The housing, maintenance, and care of the animals met or exceeded all requirements for primate husbandry. Blood samples from these monkeys were taken approximately weekly before and after inoculation and serum alanine amino transferase (ALT) levels were tested for biochemical evidence of hepatitis [Tsarev et al., 1992].

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Reverse-Transcriptase-Polymerase Chain Reaction (RT-PCR)

For biochemical detection of the HEV genome, 0.2 ml of the diluted fecal sample was extracted. The RNA extraction procedure and RT-PCR with two sets of "nested" primers were performed as previously described in detail [Tsarev et al., 1993a].

HEV Antigens

Two major proteins with molecular weights of approximately 75 kDa and 55 kDa are expressed in insect cells infected with the 63-2-IV-2 recombinant baculovirus containing the complete ORF-2 region of the Pakistani SAR-55 strain of HEV [Tsarev et al., 1993b]. Although both proteins reacted with anti-HEV by Western blots, the partially purified 55 kDa protein was shown to form virus-like particles more efficiently than the partially purified 75 kDa protein (Tsarev et al., unpublished data). Therefore, the 55 kDa protein was used as the insect cell-expressed HEV antigen in ELISA.

Two HEV antigens expressed in bacteria (3-2 (Mexico) [Goldsmith et al., 1992] and SG3 (Burma) [Yarborough et al., 1993]) were used in this study. The antigens were fusion proteins of the 26 kDa glutathione-S-transferase (GST) and either the antigenic sequence 3-2 (M) consisting of 42 amino acids located six amino acids upstream of the C-terminus of ORF-2 [Yarborough et al., 1991] or the 327 C-terminal amino acids of ORF-2 (B) [Yarborough et al., 1993].

ELISA for Anti-HEV

Sixty ng per well of the 55 kDa protein or 200 ng per well of the fusion antigens in carbonate buffer (pH 9.6) were incubated in wells of a polystyrene microtiter assay plate (Dynateck, S. Windham, ME) for 2 h at 37°C. Plates were blocked with phosphate buffered saline (PBS) containing 10% fetal calf serum and 0.5% gelatin. Serum samples were diluted 1:100 in blocking solution. Peroxidase-conjugated goat anti-human IgM (Zymed, San Francisco, CA) diluted 1:1,000 or 1:2,000, or peroxidase-labelled goat anti-human immunoglobulin diluted 1:1,000 was used as the detector antibody.

In all of the ELISAs except those for the two orally inoculated monkeys, cyno-387 and cyno-392, the 55 kDa and the fusion antigens were tested concurrently in the same laboratory so that the only variable was the antigen used. Criteria for scoring positive reactions in anti-HEV ELISA with the 55 kDa antigen were the same as used previously [Tsarev et al., 1993b]: an optical density (OD) value ≥ 0.2 and also greater than twice that of a pre-inoculation serum sample for the same animal. In addition, since both antigens expressed in bacteria were fusion proteins with GST, the OD of a sample tested with these antigens had to be three times higher than that obtained with non-fused GST in order to be considered positive [Goldsmith et al., 1992].

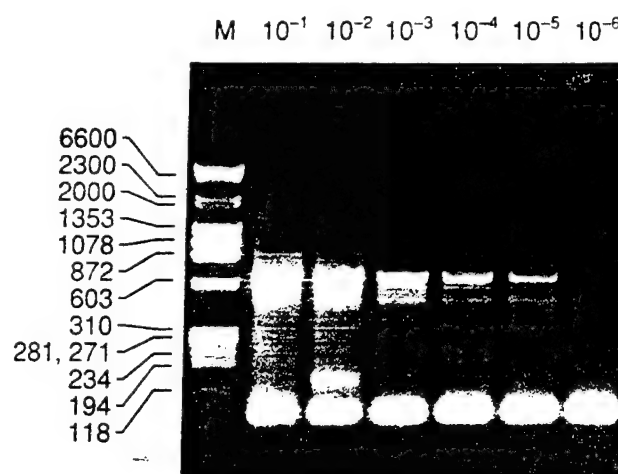


Fig. 1. Ethidium bromide stained 2% agarose gel. Separation of PCR products (predicted length about 640 b.p.) obtained from extracts of serial ten-fold dilutions (indicated for each column) of the SAR-55 HEV 10% fecal suspension. (M), DNA size markers.

RESULTS

Determination of HEV Genome Titer

Ten-fold dilutions of the fecal suspension were extracted and RT-PCR amplification was performed to determine the highest dilution in which HEV genomes could be detected. The HEV genome was detected by ethidium bromide staining of RT-PCR products in all dilutions of the standard HEV feces in the range from 10^{-1} to 10^{-5} (Fig. 1). Although the RT-PCR method with nested primers is not particularly quantitative, we observed a decrease in the amount of the specific PCR product at higher dilutions. The highest dilution of the 10% fecal suspension in which the HEV genome was detected was 10^{-5} . Therefore, taking into account the dilution factor, the HEV genome titer was approximately $10^{6.7}$ per gram of feces.

Biochemical Evidence of Hepatitis and Anti-HEV Response in Cynomolgus Monkeys Inoculated With Serial Dilutions of HEV

Two cynomolgus monkeys were inoculated by the intravenous route with a dilution of feces containing the SAR-55 strain of HEV, ranging from 10^{-1} through 10^{-8} . The 55 kDa and fusion antigens of HEV were directly compared in parallel ELISA tests of all the cynomolgus sera.

Both cynomolgus monkeys (377, 378) inoculated with the 10^{-1} dilution of the standard HEV fecal suspension had a pronounced increase in ALT activity at 4–5 weeks post-inoculation, indicative of hepatitis (Table I, Fig. 2A, 2B). All three antigens tested detected IgM anti-HEV in samples taken from cyno-377 3 weeks post-inoculation (Table I, Fig. 3A), but IgM anti-HEV was not detected in any samples from the second animal, cyno-378. IgG anti-HEV was detected in both animals with the 55 kDa-based ELISA, but only in cyno-377 with the ELISA based on fusion antigens. Values of

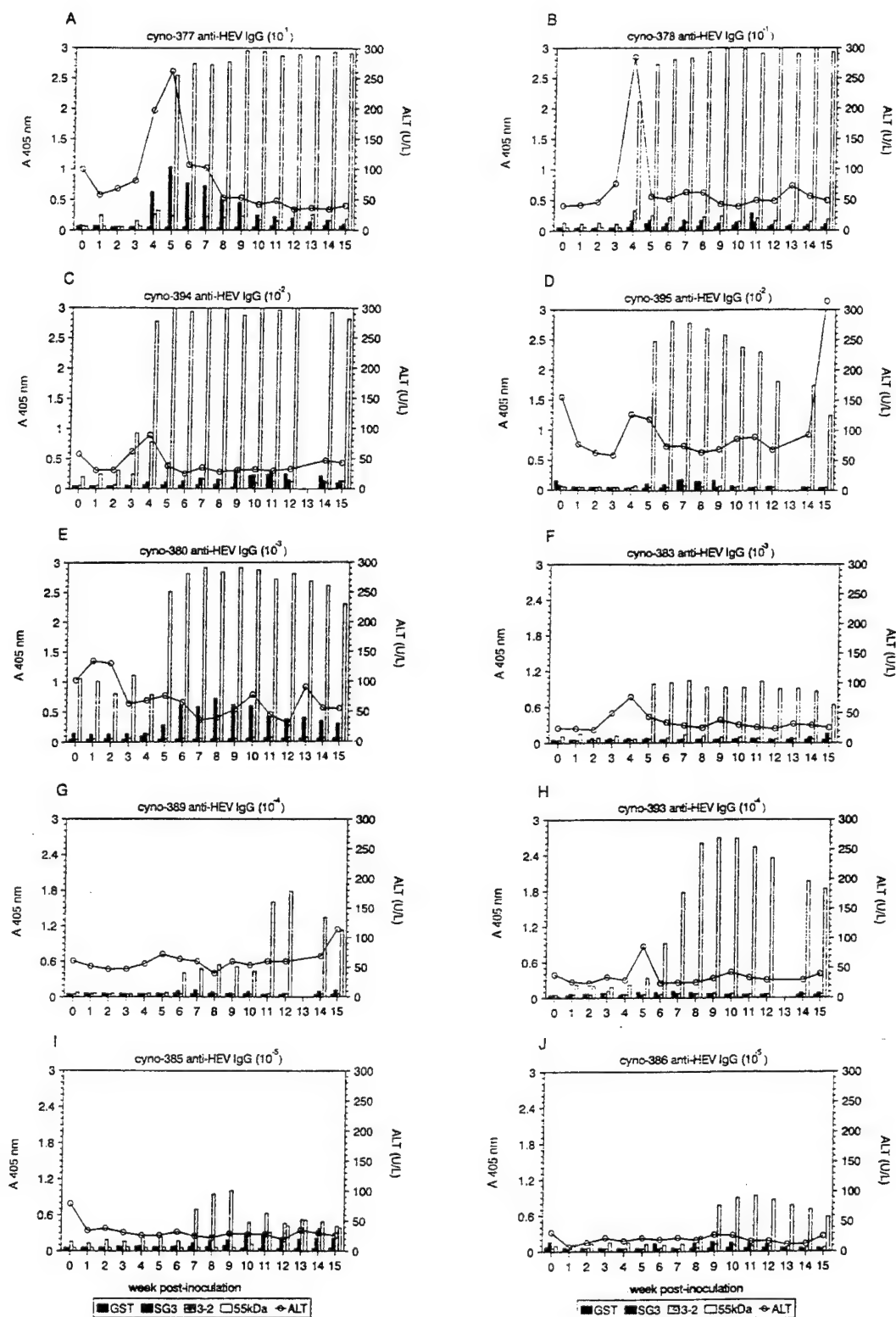


Fig. 2. (A–J) Anti-HEV IgG ELISA and ALT values for cynomolgus monkeys inoculated with ten-fold serial dilutions (indicated in parenthesis) of the 10% fecal suspension of SAR-55 HEV. Recombinant antigens used in ELISA: GST, glutathione-S-transferase; 3-2(M), fu-

sion of 3-2 epitope [Yarborough et al., 1991] and GST; SG3 (B), fusion of 327 C-terminal amino acids of ORF-2 and GST [Yarborough et al., 1993], 55 kDa, ORF-2 product directly expressed in insect cells [Tsarev et al., 1993b].

TABLE I. Summary of Biochemical and Serological Events Occurring in Cynomolgus Monkeys After Inoculation With 10^{-1} to 10^{-8} Dilutions of the Standard Stock of the SAR-55 HEV Inoculum

Cyno	Dilution of viral stock inoculum	ALT			Weeks post-inoculation anti-HEV was detected with 55 kDa antigen		Weeks post-inoculation anti-HEV was detected with fusion antigen			
		pre-inoculation mean (SD) ^a	peak week	peak value (U/L)	IgG	IgM	IgG		IgM	
							SG3	3-2(M)	SG3	3-2(M)
377	10^{-1}	76 (39)	5	264	4-15 ^b	3-7	4-10	4-5	3-4	3-5
378	10^{-1}	50 (9)	4	285	4-15	—	—	—	—	—
394	10^{-2}	62 (14)	4	89	3-15	3-10	—	4-6	—	—
395	10^{-2}	121 (21)	15	314	5-15	—	—	—	—	—
380	10^{-3}	89 (20)	1	135	5-15 ^c	—	6-15	—	—	—
383	10^{-3}	29 (8)	4	77	5-15	5-13	—	—	—	—
389	10^{-4}	60 (7)	15	114	6-15	6-8	—	—	—	—
393	10^{-4}	41 (4)	5	87	6-15	—	—	—	—	—
385	10^{-5}	59 (32)	7	56	11-15	—	—	7-15	—	—
386	10^{-5}	31 (4)	4	34	8-15	8-13	—	—	—	—
397	10^{-6}	60 (4)	8	94	—	—	—	—	—	—
398	10^{-6}	36 (3)	2	55	—	—	—	—	—	—
399	10^{-7}	102 (16)	2	93	—	—	—	—	—	—
400	10^{-7}	57 (4)	9	188	—	—	—	—	—	—
403	10^{-8}	33 (3)	2-3	49	—	—	—	—	—	—
406	10^{-8}	56 (4)	2	73	—	—	—	—	—	—
387	10^{-1} (oral) ^d	32 (4)	4	38	—	—	ND	—	ND	—
392	10^{-1} (oral) ^d	49 (6)	3	70	—	—	ND	—	ND	—

^aALT mean and standard deviation (SD) values of pre-inoculation sera.

^bThe experiment was terminated after 15 weeks.

^cThe OD values of pre-inoculation sera of Cyno-380, when tested by ELISA with 55 kDa antigen, were twice as high as the mean value of pre-inoculation sera for other cynomolgus monkeys.

^dAll ELISA tests except for Cyno-387 and Cyno-392 were performed in the same experiments.

—, not detected. ND, not done.

OD for IgG anti-HEV were significantly higher than those for IgM anti-HEV. ELISA values obtained with the 55 kDa antigen were also significantly higher than those obtained with either of the two fusion antigens (Fig. 2A, 2B). The patterns of the OD values observed in ELISA with antigens from the two sources also differed significantly. In the case of ELISA based on the fusion antigens, positive signals reached a maximum shortly after seroconversion and then waned during the 15 weeks of the experiment. In ELISA based on the 55 kDa antigen, the positive signal reached a maximum shortly after seroconversion and remained at approximately the same high level throughout the experiment (15 weeks).

Elevation in ALT activities in both monkeys (394 and 395) inoculated with a 10^{-2} dilution of the standard HEV stool suspension was significantly less pronounced at the expected time of hepatitis than in animals inoculated with the ten-fold higher dose (Table I, Fig. 2C, 2D). Cyno-395 actually had higher ALT activities prior to inoculation as well as at 15 weeks post-inoculation. The latter was probably not related to HEV infection. Weakly positive IgM anti-HEV was detected only in cyno-394 (Fig. 3B) and only with ELISA based on the 55 kDa antigen. Both animals were infected, however, since IgG anti-HEV seroconversion was detected in both animals. In cyno-394, anti-HEV IgG was first detected by the 55 kDa antigen at week 3 and 1 week later with the 3-2(M) antigen. The SG3 (B) antigen did not detect seroconversion in cyno-395 and anti-HEV IgG was detected only with the 55 kDa anti-

gen. Anti-HEV tended to diminish in titer with time in this animal.

Cyno-380 and cyno-383 were inoculated with a 10^{-3} dilution of the standard HEV fecal suspension (Table I, Fig. 2E, 2F, 3C). Cyno-380 had fluctuating ALT activities before and after inoculation; therefore, ALT levels could not be used to document hepatitis E in this animal. In Cyno-383, a slight rise of ALT activities was observed (Fig. 2F), which was coincident with seroconversion and, therefore, might be due to mild hepatitis E. IgM Anti-HEV was not detected in sera from cyno-380 with any of the three antigens. Cyno-380 seroconverted for IgG anti-HEV when tested by ELISA with SG3 (B) but not with 3-2(M) antigen. This animal had preexisting IgG anti-HEV when tested with the 55 kDa antigen, but there was a large increase in IgG anti-HEV starting at week 5 (Fig. 2E). Identification of pre-existing antibody was reported earlier in sera from another cynomolgus monkey [Ticehurst et al., 1992b; Tsarev et al., 1993b]. Seroconversion occurred at the expected time but the levels of IgG anti-HEV in samples from cyno-383 remained low and detectable only with the 55 kDa antigen.

Cyno-389 and cyno-393 were inoculated with a 10^{-4} dilution of the standard HEV fecal suspension (Fig. 2G, 2H, 3D, Table I). Neither animal had a significant rise in ALT activities, although the timing of a small but distinct ALT peak in sera of cyno-393 at week 5 (Fig. 2H) suggested borderline hepatitis. ELISA based on the SG3 (B) or 3-2(M) antigens scored both animals as negative for HEV infection. In contrast, the 55 kDa antigen

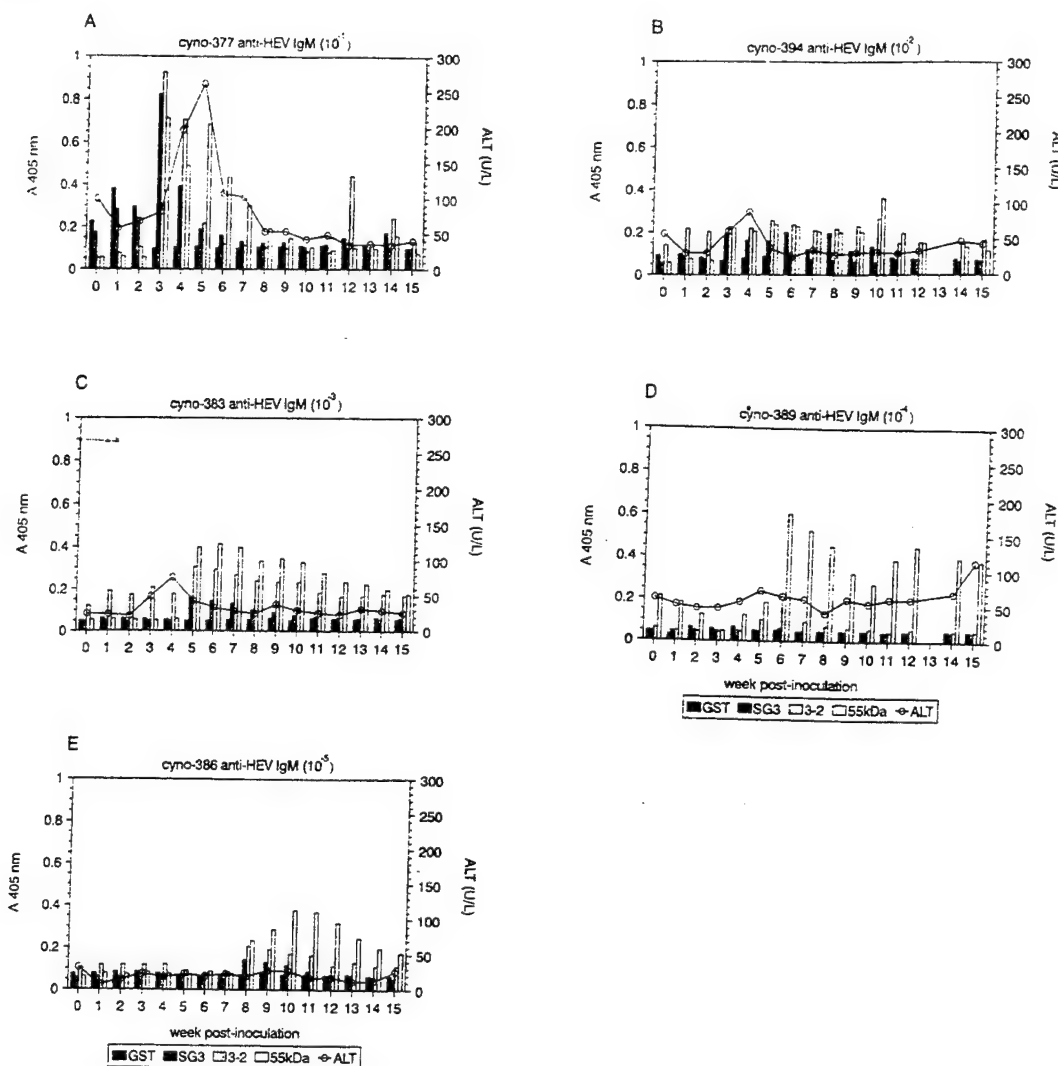


Fig. 3. (A–D) Anti-HEV IgM ELISA and ALT values (shown for comparison with data in Figure 2) for positive cynomolgus monkeys inoculated with ten-fold serial dilutions (indicated in parenthesis) of the 10% fecal suspension of SAR-55 HEV. Recombinant antigens used in ELISA: GST, glutathione-S transferase; 3-2(M), fusion of 3-2 epitope [Yarborough et al., 1991] and GST; SG3 (B), fusion of 327 C-terminal amino acids of ORF-2 and GST [Yarborough et al., 1993]; 55 kDa, ORF-2 product directly expressed in insect cells [Tsarev et al., 1993b].

detected IgM anti-HEV in sera of cyno-389 at weeks 6–8 post-inoculation (Fig. 3D) and IgG anti-HEV from week 6 through week 15 in both animals.

Neither animal inoculated with the 10^{-5} dilution of the standard fecal suspension developed a noticeable rise in ALT activities (Fig. 2I, 2J, Table I), but, in cyno-386, IgM and IgG anti-HEV were detected at weeks 8–13 and 8–15 respectively with the 55 kDa antigen (Fig. 2J, 3E). Cyno-385 anti-HEV IgG was detected with the 55 kDa and the 3-2(M) antigen but not with SG3 (B) antigen. In contrast to previous patterns, IgG anti-HEV was detected with a fusion antigen 4 weeks earlier and at higher levels than with the 55 kDa antigen.

None of the animals inoculated with dilutions of the standard HEV fecal suspension in the range of 10^{-6} – 10^{-8} developed antibody to any of the three HEV antigens. Increased ALT activities were not observed in those animals, except for one rather prominent peak of ALT activity at week 9 in cyno-400 (Table I). However, the absence of seroconversion in this animal indicated that this peak probably was not related to HEV infection.

Two cynomolgus monkeys (387 and 392) were inoculated orally with the 10^{-1} dilution of the 10% fecal suspension. Neither monkey was infected since ALT levels did not rise and ELISA carried out with the

3-2(M) and 55 kDa antigens did not detect seroconversion to HEV (Table I).

DISCUSSION

Infectivity of the HEV SAR-55 Viral Stock

Knowledge of the infectious titer of inocula is critical for interpretation of much of the data obtained in experimental infections of animal models. However, until now the infectious titer of an HEV viral stock has not been reported. In this study we used cynomolgus monkeys since they are a useful laboratory model for HEV infection [Balayan et al., 1983; Bradley et al., 1987; Uchida et al., 1990; Ticehurst et al., 1992b; Tsarev et al., 1993a]. We found that a 10^{-1} dilution of a standard 10% fecal suspension of SAR-55 was not able to produce infection after oral inoculation of cynomolgus monkeys. However, when inocula were administered intravenously, all decimal dilutions up to and including a 10^{-5} dilution were infectious for cynomolgus monkeys. The calculated 50% infectivity titer for cynomolgus monkeys was approximately $10^{6.8}$ per gm of feces. Comparable differences between infectious titers of inocula administered orally versus intravenously have also been found for hepatitis A virus [Purcell et al., unpublished data].

Only those dilutions that were shown by RT-PCR to contain the HEV genome were infectious for cynomolgus monkeys. Therefore, the infectivity titer of the standard fecal suspension and its genome titer as detected by RT-PCR were approximately the same. A similar correlation between RT-PCR and infectivity titer was found for one strain of hepatitis C virus [Cristiano et al., 1991; Farci et al., 1991; Bukh et al., 1992].

Serological evidence for HEV infection was found in all animals inoculated with decimal dilutions of the fecal suspension through 10^{-5} ; none of the animals receiving higher dilutions had such evidence. Prominent hepatitis, as defined by elevated ALT, was observed only in the two monkeys infected with the 10^{-1} dilution. Significantly lower elevations of ALT activities were observed in some animals inoculated with higher dilutions of the fecal suspension while, in others, elevations were not found. Considered alone, these low ALT rises were not diagnostic of hepatitis. However, the coincidence of seroconversion and appearance of these ALT peaks suggests the presence of mild hepatitis in these animals. Anti-HEV IgG seroconversion was detected in all animals inoculated with dilutions of fecal suspension ranging from 10^{-1} – 10^{-5} . A tendency toward lower levels of IgG anti-HEV and delayed seroconversion was observed in animals inoculated with higher dilutions of the stock.

Detection of IgM anti-HEV was not as efficient as detection of IgG anti-HEV and OD readings for IgM anti-HEV were often only slightly above the cutoff value. One or both of the following reasons could explain the lower ELISA readings. First, in cynomolgus monkeys the IgM anti-HEV response might normally be significantly lower than the IgG response. Second, it

could be that the detector antibodies used for the two classes of cynomolgus immunoglobulins differed in sensitivity. That used for IgM and that for IgG were raised against heterologous immunoglobulins, human IgM and IgG respectively. In anti-HEV ELISA of human sera, detection of anti-HEV IgM was significantly more efficient than when cynomolgus monkeys were tested [Yarborough et al., 1993; Dawson et al., 1992; Bryan et al., 1993; Tsarev et al., 1993a]. When measured with homologous anti-human detector antibodies and the 55 kDa antigen ELISA, typical anti-HEV IgG and anti-HEV IgM titers in humans [Bryan et al., 1993] were comparable (1:1,000–1:10,000) during acute hepatitis, in contrast to the 10- to 100-fold lower anti-HEV IgM titers found here for the cynomolgus monkeys.

We found that levels of ALT and anti-HEV responses in cynomolgus monkeys depended on the amount of the virus inoculated into the animal and both indicators of infection diminished in value as the inoculum was further diluted. At higher dilutions of inoculum, hepatitis became milder and HEV infection could be detected only by seroconversion. Inapparent infection has also been reported for humans [Arankalle et al., 1988].

Comparison of the ORF-2 HEV Antigens Expressed in Insect Cells and Bacteria for Use in ELISA

Two partial regions of ORF-2 expressed as fusion proteins in bacteria and a product of the complete ORF-2 expressed in insect cells were used in this study for anti-HEV ELISA. One fusion protein, 3-2(M), contained region 3-2 [Yarborough et al., 1991] and the other, SG3 (B), contained the 327 C-terminal amino acids of ORF-2 including the 3-2 epitope. The complete ORF-2 of the Pakistani strain expressed from a baculovirus vector [Tsarev et al., 1993b] in insect cells accumulated as two major proteins with molecular mass of 75 kDa and 55 kDa. Only the 55 kDa protein was used in this study.

In general the 55 kDa Pakistani ORF 2 antigen was more efficient than either the 3-2(M) or SG3 (B) antigen for detecting IgM and IgG anti-HEV in cynomolgus monkeys infected with the Pakistani strain of HEV. In this study, for all animal sera except those from cyno-385, detection of IgG or IgM anti-HEV by ELISA was more efficient with the 55 kDa antigen than with either the 3-2(M) or SG3 antigen. ELISA with the 55 kDa antigen produced internally consistent and reproducible results, detecting IgG anti-HEV in all ten animals inoculated with a fecal dilution of 10^{-5} or lower. The magnitude of ELISA signals also decreased as the inoculum was diluted. The fusion antigens did not produce consistent results between the pairs of animals. Only one of each pair of animals inoculated with the 10^{-1} , 10^{-2} , 10^{-3} , or 10^{-5} dilution showed seroconversion to IgG anti-HEV, and only a single seroconversion for IgM anti-HEV was detected with these antigens. Neither of the animals inoculated with the 10^{-4} dilution of the original inoculum seroconverted to either of the two

fusion antigens even though sera from one animal (cyno-393) had sustained high levels of anti-HEV IgG when assayed with the 55 kDa antigen. Although, as discussed above, ELISA for IgM anti-HEV was significantly less sensitive than ELISA for cynomolgus IgG anti-HEV, the 55 kDa antigen was able to detect anti-HEV IgM in more animals than the 3-2(M) or SG3 (B) antigen. Overall, a definitive conclusion about the infectious titer of the Pakistani viral inoculum used in this study could not be made with the combined data from the 3-2(M) and SG3 (B) based ELISA but could be made with data obtained with the 55 kDa Pakistani ELISA alone.

Cyno-385 is the only animal in which seroconversion to anti-HEV was detected earlier with a fusion antigen than with the 55 kDa antigen. The difference in anti-HEV IgG detection between the two test results was 4 weeks. These data suggest the presence of a distinct epitope in the 3-2(M) antigen recognized by this animal that is absent in the larger 55 kDa and SG3 (B) antigens. When total insect cell lysate, which contained both complete ORF-2 (75 kDa) and 55 kDa proteins, was used as antigen to retest these samples, the results were the same as when 55 kDa was used alone. This finding suggests that the 55 kDa protein may not lack 3-2 epitope amino acids but rather that the conformation of the 3-2 epitope sequence differed among all three antigens used in this study. It is interesting that despite the fact that antigen SG3 (B) contained a longer portion of ORF-2 and included the entire sequence of epitope 3-2, it did not detect more positive sera than the 3-2(M) antigen. In fact, anti-HEV IgG was detected in cyno-385 only with the smallest antigen, 3-2(M). Of four animals for which seroconversion was detected by the fusion antigens, in only one case did both antigens react. In the other three animals, the shorter antigen 3-2(M) detected anti-HEV in two of the three, while the longer antigen SG3 (B) detected anti-HEV only in one animal.

One explanation for the difference observed in sensitivity of the ELISAs could be that the antigenic structure of the capsid protein of the Mexican strain used for the 3-2(M) or that of the Burma strain used for the SG3 (B) antigen differed from that of the SAR-55 strain used for the 55 kDa antigen. However, this explanation seems unlikely for several reasons. All of the existing data indicate that there is only one serotype of HEV. The 55 kDa antigen derived from the sequence of the SAR-55 Pakistani strain was equally able to detect anti-HEV in animals inoculated with the highly divergent Mexican strain of HEV or with the Pakistani HEV strain [Tsarev et al., 1993b]. In addition, antigen 3-2(M) based on the Mexican strain of HEV is equally efficient for detection of anti-HEV in human sera of Mexican or Asian origin as is antigen 3-2 (B) based on the Burmese strain of HEV [Yarborough et al., 1991; Goldsmith et al., 1992]. However, epitopes found in ORF-3 of HEV are more strain-specific [Yarborough et al., 1991]. Therefore, it is possible that the increased

sensitivity of the 55 kDa antigen ELISA is a function of its larger size and/or conformation.

In conclusion, in this study we have characterized a standard fecal suspension of a Pakistani strain of HEV for its ability to infect cynomolgus monkeys and have determined its genomic and infectivity titers. This strain of HEV was isolated from a well characterized outbreak of hepatitis E [Iqbal et al., 1989; Ticehurst et al., 1992a; Bryan et al., 1993] and has been characterized extensively biologically by experimental infection of different primate species [Tsarev et al., 1993a; Tsarev et al., 1993b]. In addition, we have determined the complete sequence of the SAR-55 genome [Tsarev et al., 1992] and have shown that it is more closely related to many other strains of HEV recovered from Asia than are the Burmese or Mexican strains [Tsarev et al., 1992; Yin et al., 1993]. This extensive characterization of SAR-55 makes it a logical choice for prototype status in further investigations of HEV, particularly in studies of vaccine development.

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Association of Hepatitis E Virus With an Outbreak of Hepatitis in Pakistan: Serologic Responses and Pattern of Virus Excretion

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Hepatitis E virus (HEV), a positive-strand RNA agent, has been associated with enterically transmitted non-A, non-B hepatitis in Asia, Africa, and Mexico. To evaluate the role of HEV in an outbreak of hepatitis in Pakistan, we used immune electron microscopy to detect 1) antibody to HEV, for evidence of infection, and 2) virus, to determine the pattern of HEV excretion. Paired sera from 2 patients were assayed for antibody by using reference HEV: one seroconverted, an atypical finding for HEV infections; the other had high levels of anti-HEV in both sera. Virus particles with the size (29 × 31 nm) and morphology of HEV were detected in feces from 10 of 85 patients and serologically identified as HEV by using reference antibodies from an HEV-infected chimpanzee. One of these HEV-containing specimens was collected 9 days before the onset of jaundice; it was among feces from 38 outpatients with nonspecific symptoms and biochemical hepatitis, 12 of whom subsequently developed jaundice. The other 9 feces with HEV were among 36 collected within 7 days of the onset of acute icteric hepatitis; all 11 feces from days 8 to 15 were negative for HEV. Fecal concentrations of HEV appeared to be lower than those of many enteric viruses: only one specimen contained as many as 5 particles per EM grid square. It is concluded that HEV was etiologically associated with the epidemic and was predominantly excreted at very low levels during the first week of jaundice.

KEY WORDS: enterically transmitted non-A, non-B (ET-NANB) hepatitis; epidemic, immune electron microscopy (IEM), antibody, virus particles

INTRODUCTION

Epidemics of enterically transmitted non-A, non-B (ET-NANB) hepatitis have been recognized in Asia, Africa, and Mexico [for reviews, see Balayan, 1987; Bradley, 1990a,b; Bradley et al., 1988b; Purcell and Ticehurst, 1988; Ticehurst, 1991]. Although ET-NANB hepatitis has many characteristics of hepatitis A, its incubation period appears to be longer, cholestasis is more prominent, the mortality rate in pregnant women is unusually high (10–20%), and secondary cases are rare.

The only identified agent of ET-NANB hepatitis has been unofficially designated as hepatitis E virus (HEV) [Asher et al., 1990; Balayan et al., 1990; Bradley, 1990b; Humphrey et al., 1990; Purcell and Ticehurst, 1988; Reyes et al., 1990; Tam et al., 1991; Tsarev et al., 1992; Ticehurst, 1991; Yarbough et al., 1991; Zuckerman, 1990]. Although HEV is not well characterized, it appears to be somewhat distinctive. The viral genome is a positive-sense, 7.6 kb, 3'-polyadenylated RNA, but there is little similarity among partial amino acid sequences of HEV and caliciviruses, Norwalk virus, or other viruses [Jiang et al., 1990; Neill, 1990; Neill et al., 1991; Reyes et al. 1990] (Ticehurst, Neill, and Reyes, unpublished data). Like certain caliciviruses [for reviews, see Cubitt, 1989; Schaffer, 1979], HEV has a sedimentation coefficient of 183 S [Bradley et al., 1988a] and was detected at densities of 1.29 g/cm³ in potassium tartrate/glycerol [Bradley, 1990a,b] and 1.35 g/cm³ in CsCl [Balayan et al., 1983]; it was of interest

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that Bradley et al. [1988b] could not recover HEV from CsCl. Reported diameters of HEV particles (27 to 34 nm) and caliciviruses are similar [Arankalle et al., 1988; Balayan et al., 1983, 1990; Bradley, 1990a,b; Bradley et al., 1987, 1988a,b; Purcell and Ticehurst, 1988; Ticehurst, 1991]. HEV has electron-lucent areas and outer indentations, somewhat like Norwalk virus, but these are less prominent than the typical surface features of caliciviruses.

HEV was first associated with ET-NANB hepatitis by Balayan et al. [1983], who used immune electron microscopy (IEM) to observe virus in acute-phase feces from Soviet Central Asia. Although HEV was subsequently identified in feces from other regions and, in addition, antibody to HEV (anti-HEV) was detected in many cases of ET-NANB hepatitis, studies of hepatitis E have been hindered by two characteristics of HEV infections. First, it was usually impossible to prove that a patient was recently infected because the level of anti-HEV during acute jaundice was at least as high as that during convalescence. However, confirmatory evidence of an etiologic role for HEV was obtained by using pre-exposure and acute-phase sera to detect seroconversions in a few natural cases and in experimentally inoculated primates [Arankalle et al., 1988; Bradley et al., 1987, 1988a,b; Krawczynski and Bradley, 1989; Purcell and Ticehurst, 1988]. Second, it appears that HEV is labile or excreted at such low levels that approximately 2,200 human fecal specimens were screened to find 2 with high concentrations of HEV [Bradley et al., 1988a,b]. Because HEV has not been frequently detected, the relationship between excretion of HEV and onset of symptoms is not well defined.

We evaluated the role of HEV in an outbreak of ET-NANB hepatitis at a college in Sargodha, Pakistan. IEM was used to demonstrate that HEV was etiologically associated with this outbreak and to determine the pattern of HEV excretion.

MATERIALS AND METHODS

Patients

The Sargodha outbreak of ET-NANB hepatitis, in which 133 patients were hospitalized, appeared to result from contamination of the water supply to several dormitories by raw sewage [Iqbal et al., 1989]. On March 20, 1987, single specimens of feces and serum were collected from the residents of the dormitories. Most sera had elevated levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), or total bilirubin. Some of those who were exposed to the contaminated water had symptoms consistent with a prodromal phase of illness or anicteric hepatitis. Several of these latter individuals subsequently developed frank jaundice. Convalescent-phase sera were obtained 31 days after the first specimens, on April 18.

Specimens

Sera and feces were usually stored and transported at $\leq -70^{\circ}\text{C}$. Portions of some specimens were lyophilized and then stored and transported at 4°C prior to

reconstitution with water. Dilutions of sera were made in phosphate-buffered saline, pH 7.4 (PBS) (Quality Biological, Gaithersburg, MD, USA). Fecal suspensions (10% wt/vol, in PBS) were prepared as described by Arankalle et al. [1988] and then stored at -80°C in aliquots of <2 ml to avoid repeated freezing and thawing.

IEM

Technique. IEM was performed as previously described [Arankalle et al., 1988; Feinstone et al., 1973; Kapikian et al., 1976; Thornhill et al., 1975] with several modifications. In many experiments, the final dilution of serum or plasma was 1:50; for detecting virus, however, sources of antibody were often used at a higher final dilution (1:250 or 1:500) to increase the likelihood of forming larger, more readily detected immune complexes [Dienstag et al., 1976; Elkana and Guttman-Bass, 1983]. Each suspension of feces (160 μl) or bile (40 μl ; see below and Table I) was mixed with diluted serum and sufficient PBS to give a final volume of 1 ml in a 10×75 mm Pyrex tube (DuPont Instruments, Wilmington DE, USA) and incubated at room temperature for 1 hr and then at 4°C overnight. After centrifugation at 36,000g and 4°C for 90 min, the pellet was resuspended in 40 to 800 μl of filtered (0.22 μm Millex-GS, Millipore, Bedford MA, USA) water, sufficient to produce a slightly turbid suspension. A portion of this resuspended pellet was negatively stained with an equal volume of 3% wt/vol sodium phosphotungstate, pH 7.2 (PTA) or, to improve the spreading of specimens, of 25 $\mu\text{g}/\text{ml}$ of Bacitracin (Upjohn, Kalamazoo MI, USA) in 2% wt/vol PTA [Gregory and Pirie, 1973] and applied to a carbon-Formvar-coated grid.

Grids were examined and photographed at $50,000 \times$ in an electron microscope (JEM-100B, Japan Electron Optics Laboratory, Tokyo, Japan; or EM-10, Carl Zeiss, Oberkochen, Germany). Magnification was calibrated by using a diffraction-grating replica containing 2,160 lines/mm (Ernest F. Fullam, Rochester NY, USA). Three to 15 grid squares were scanned for virus particles. Antibody was rated on a scale of 0 to 4+, where 0 represented no antibody and 4+ indicated heavy antibody that nearly obscured the particle. A rating of 0–1+, indicating a very light coating of antibody, or antibody-like material, was interpreted as negative.

Screening for virus in single fecal suspensions and antibody in pooled sera. We initially evaluated specimens collected within 3 days of the onset of jaundice or dark urine from 10 Sargodha patients to determine if viruses or antibody were present. Equal volumes of these patients' sera were pooled and assayed with individual fecal suspensions; the final dilution of each serum was 1:500. None of these preparations was examined under random code.

A fecal specimen was considered to contain virus when at least 3 single antibody-coated virus particles or 3 aggregates of antibody-coated particles were ob-

TABLE I. Tests of Specimens From Sargodha, Pakistan: IEM With HEV Reagents

Purpose of test	Specimens tested	Source of	HEV reagents		Antibody rating ^a		
			Contained in	Origin of HEV	Pre	Acute	Conv
Detect anti-HEV	Sera	HEV particles	TK-021 feces Cyno 9A97 bile	Kathmandu, Nepal, 1987 Telixtac, Mexico, 1986	NA	0 0-1+	2+ 3+
	Sar-12		Sar-55 feces Cyno 9A97 bile	Sargodha, Pakistan, 1987 Telixtac, Mexico, 1986	NA	3-4+ 3-4+	2-3+ ^b 3-4+
	Feces	Anti-HEV	Chimp 945 plasma	Kolhapur, India, 1981	0	3-4+	NT
Identify virus as HEV							

^a Ratings by IEM of pre-inoculation (Pre), acute-phase, and convalescent-phase (Conv) sera or plasma when tested by IEM at a final dilution of 1:50; NA, not available; NT, not tested.

^b Convalescent-phase antibody was detected only after the code was broken because the concentration of HEV in the IEM preparation was very low.

served. If only one or 2 such particles were detected, the specimen was considered positive if at least one particle or aggregate was observed when the test was repeated.

Detection of anti-HEV. To determine if individual sera from the Sargodha pool (see above) contained anti-HEV, we used known sources of infectious HEV: 1) feces, designated TK-021, from Nepal (B. Innis et al., unpublished data); 2) bile from a cynomolgus monkey (*Macaca fascicularis*; designated Cyno 9A97) that was experimentally inoculated with the Telixtac-14 virus [Bradley et al., 1988a; Velázquez et al., 1990] (Ticehurst et al., submitted for publication); and 3) feces from Sargodha patient Sar-55 (Table I; see Results and Discussion). The bile was used at a dilution of 1:10 in 0.05 M Tris-HCl (pH 7.3) / 0.15 M NaCl (Ticehurst et al., submitted for publication). These IEM reactions were read under code.

Detection of HEV particles. Plasma from an HEV-infected chimpanzee (*Pan troglodytes*) was used to determine if virus in Sargodha feces could be serologically identified as HEV. Chimp 945 was inoculated with feces from a patient with ET-NANB hepatitis in India (collected in Kolhapur during 1981); it subsequently developed hepatitis and antibody to HEV from India (Ahmedabad, 1984), Mexico (1986), and Nepal (1987) [Arankalle et al., 1988; Purcell and Ticehurst, 1988; Ticehurst, 1991] (Innis et al., unpublished). Chimp 945 plasma was used in coded IEM preparations at a final dilution of 1:50 or 1:500.

Size of virus particles. Diameters of viruses were determined by making 3 measurements per particle, including the shortest and longest dimensions, on the negative plates. Virus size is reported as mean short diameter \times mean long diameter, \pm standard deviations, in accordance with measurements of other viruses [Feinstone et al., 1973; Kapikian et al., 1976].

RESULTS

Screening for Virus in Single Fecal Suspension and Antibody in Pooled Sera

We examined early acute-phase specimens from 10 patients in an outbreak of ET-NANB hepatitis at Sargodha, Pakistan. Virus particles that morphologically resembled HEV were detected in 6 of the 10 feces. These particles were heavily coated with antibody (4+ ratings; Fig. 1A,B) that often had the staple-like morphology thought to be characteristic of IgM [Almeida and Waterson, 1969]. The antibody rating appeared to be lower (2-3+) when a higher final dilution (1:500) of the pool of 10 sera was tested (data not shown). We concluded that this IgM-like antibody was derived from the pooled sera, an indication that at least one of the 10 patients had been infected, probably recently, with the observed virus.

Detection of Anti-HEV

Paired sera from 2 Sargodha patients were tested for antibody to known HEV (Table I); the acute-phase specimens were part of the serum pool used to screen for virus and antibody (see above). Patients Sar-5 and

Sar-12 donated these serum pairs 2 and 31 days and 3 and 32 days, respectively, after the onset of jaundice. Anti-HEV was not detected in the Sar-5 acute-phase serum, an unusual finding [Arankalle et al., 1988; Bradley et al., 1988a,b; Purcell and Ticehurst, 1988]. Because of the rise in antibody rating between illness and convalescence (Fig. 2), we concluded that patient Sar-5 was infected with HEV during the epidemic. In contrast, high levels of anti-HEV were detected in both sera from patient Sar-12 (Fig. 3), so a conclusion could not be made about when this patient was infected. However, in the IEM preparation from the acute-phase serum, HEV particles were often coated with IgM-like antibody (Fig. 3A,C) [Almeida and Waterson, 1969] that was not detected during convalescence. Sera from other patients were not tested because the quantity of HEV was limited.

Detection of HEV Particles

Virus particles in feces from Sargodha patient Sar-55 were not coated with antibody when incubated with pre-inoculation plasma from Chimp 945, but they did react with antibody in plasma collected during the acute phase of HEV infection (Fig. 4, Table I). In addition, virus particles in certain other Sargodha feces, including Sar-12, were coated with antibody when tested with Chimp 945 acute-phase plasma (Fig. 1C-E). IEM results were similar when this plasma, instead of the pool of acute-phase Sargodha sera (see above), was used as the source of antibody for detecting virus.

HEV particles were detected in a total of 10 single fecal suspensions from 95 individuals in Sargodha. The concentration of HEV was very low: <1 to approximately 5 particles per EM grid square. HEV particles

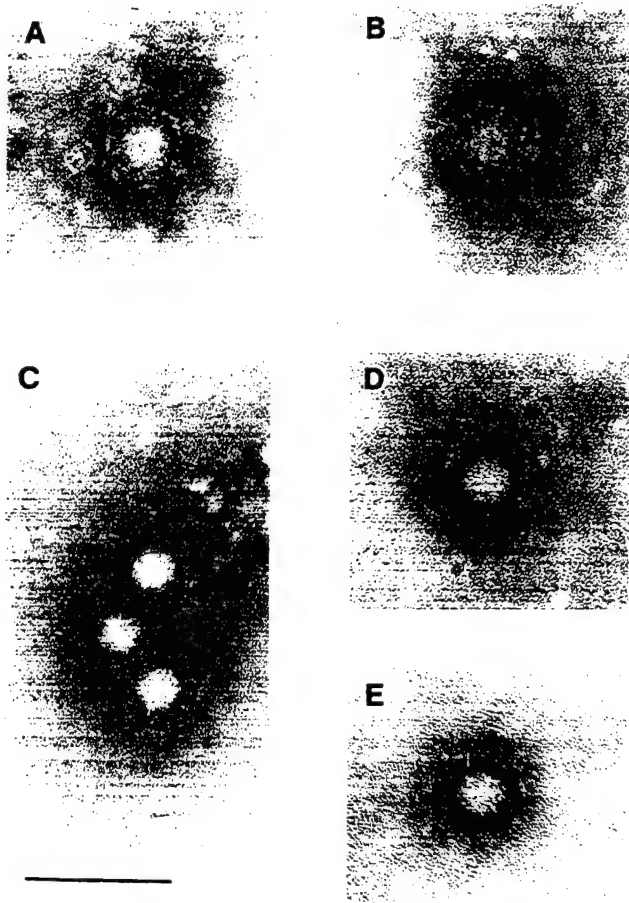


Fig. 1. Virus particles in feces from Sargodha, Pakistan, as detected by IEM. A and B: A pool of 10 acute-phase sera from Sargodha patients (see Materials and Methods) was used as a final dilution of 1:50 as a source of antibody. A: Patient Sar-50, antibody rating: 4+, with a staple-like appearance thought to be characteristic of IgM [Almeida and Waterson, 1969]. B: Patient Sar-18, antibody rating: 4+, IgM-like. C-E: Acute-phase Chimp 945 plasma was used as a source of anti-HEV at a final dilution of 1:500. C: Patient Sar-19, antibody rating: 3-4+. D: Patient Sar-4, antibody rating: 3-. E: Patient Sar-12, antibody rating: 3+. Bar = 100 nm.

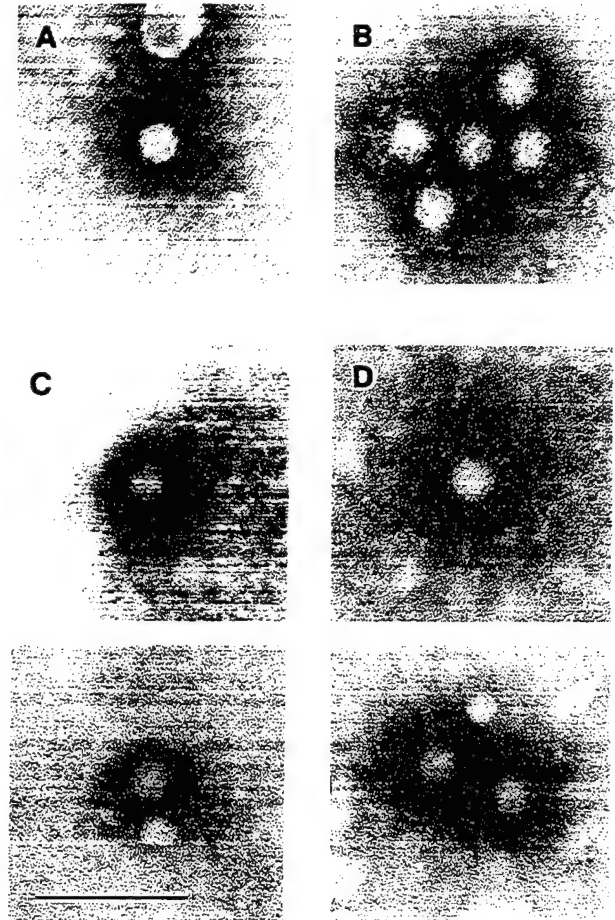


Fig. 2. Seroconversion to HEV detected by testing of sera from patient Sar-5. TK-021 feces (A and B) or Cyno 9A97 bile (C and D) was used as a source of HEV. A: Acute-phase serum, antibody rating: 0 (because of absence of antibody, it is not certain that this, the only such particle observed, is HEV). B: Convalescent phase, antibody rating: 2-. C: Acute phase, antibody rating: 0-1+ (because of minimal, if any, antibody, it is not certain that these particles are HEV although many such particles were observed). D: Convalescent phase, antibody rating: 3+. Bar = 100 nm.

were detected in suspensions prepared from lyophilized or frozen feces, but the concentration of HEV was too low to determine which method of storage was better for preserving the virus.

Morphology and Size of HEV Particles

The appearance of HEV particles from Sargodha (Figs. 1, 3, 4) was similar to that of previously identified HEV particles (Figs. 2, 3) [Arankalle et al., 1983; Balayan et al., 1983; Bradley et al., 1988a]. These particles had more surface structure than picornaviruses, but somewhat less than Norwalk virus and markedly less than typical caliciviruses. Thick-shelled, apparently disrupted particles were occasionally seen that were distinct from typically thin-shelled empty particles of picornaviruses (Fig. 4B). The mean diameters of 65 HEV particles from Sargodha were $29 \pm 1.4 \times 31 \pm 1.5$ nm, similar to those of HEV from Mexico (29×31 nm in Cyno 9A97 bile) and Nepal (27×31 nm in TK-021 feces) [Ticehurst, 1991].

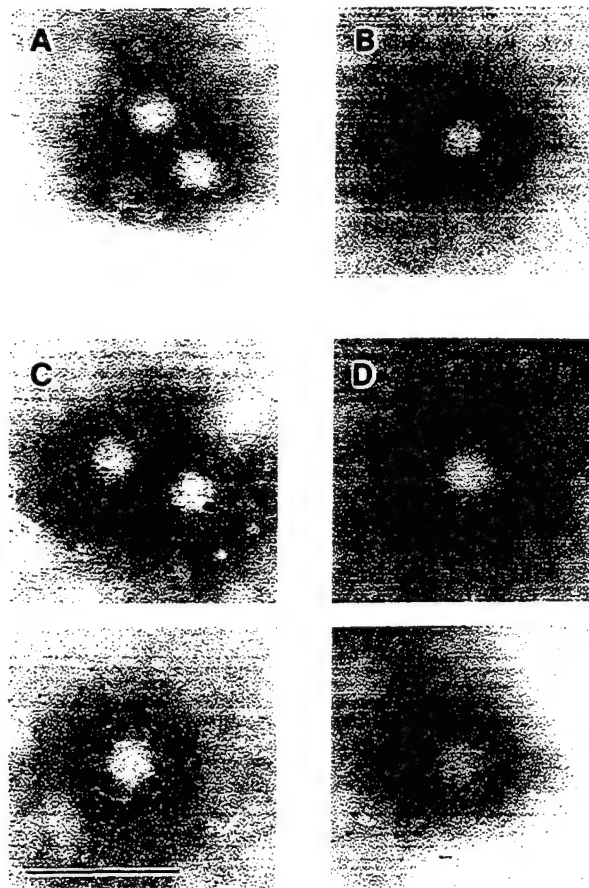


Fig. 3. Anti-HEV detected in sera from patient Sar-12. Sar-55 feces (A and B) or Cyno 9A97 bile (C and D) was used as a source of HEV. A and C: Acute-phase serum, antibody rating: 3-4+. IgM-like. B: Convalescent phase, antibody rating: 2-3+. D: Convalescent phase, antibody rating: 4+. Bar = 100 nm.

Pattern of HEV Excretion

HEV was detected in fecal suspensions from 10 of 85 patients in Sargodha who had objective evidence of hepatitis (Table II). Among 38 outpatients with anicteric hepatitis, 12 of whom were subsequently admitted to the hospital with jaundice, only one (3%) excreted detectable HEV. This individual had elevated levels of ALT, AST, and bilirubin and was hospitalized 9 days after the specimen with HEV was collected. The remaining 26 outpatients were presumed to have remained anicteric because they had been instructed to return if jaundice developed. In contrast, 9 of 47 (19%) acute-phase inpatients excreted detectable HEV; all 9 specimens were obtained within 7 days of the onset of jaundice or dark urine and represent 25% of the feces

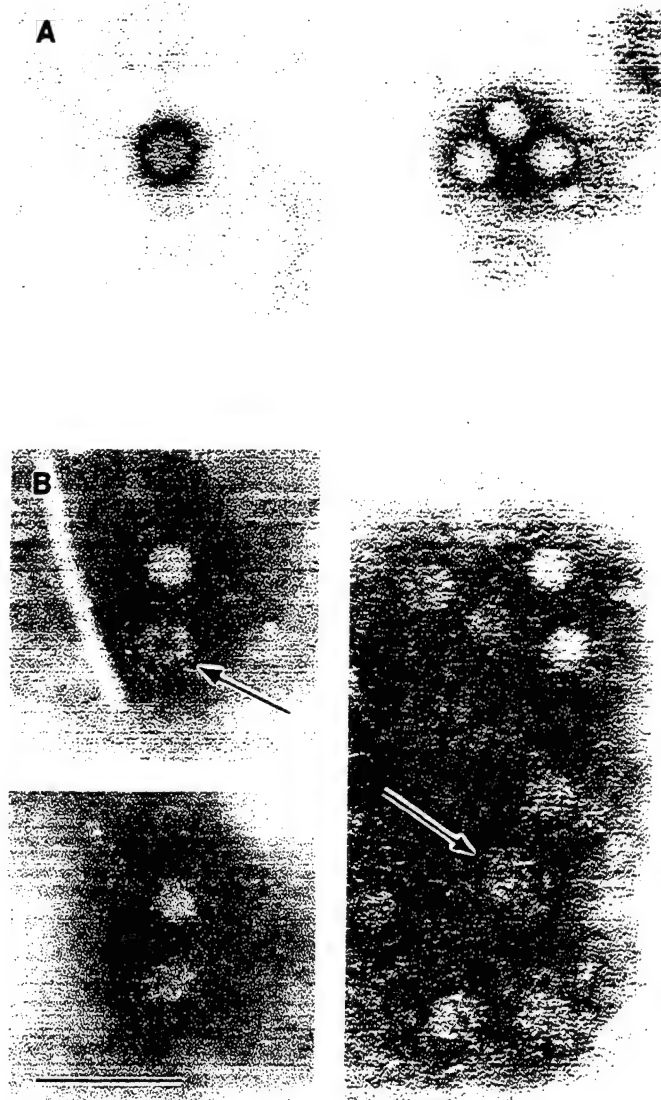


Fig. 4. Identification of HEV in feces from Sargodha patient Sar-55. A: Pre-inoculation plasma from Chimp 945, antibody rating: 0 (because of the absence of antibody, it is not certain that these particles are HEV). B: Acute phase, antibody rating: 3-4+. Several of the particles appear to be disrupted or "empty" (arrows). Bar = 100 nm.

TABLE II. Relationship Between Clinical Findings and Excretion of HEV From Patients in Sargodha, Pakistan, With Biochemical Evidence of Hepatitis

Findings ^a	Phase of illness ^b	HEV in feces ^c
Anicteric		1/38 ^d
	Indeterminate	0/26 ^e
	Prodromal	1/12 ^f
Acute icteric hepatitis		9/47 ^{d,e}
	≤ 7 days	9/36 ^{f,g}
	8-15 days	0/11 ^g

^aSigns and symptoms when fecal specimen was collected. Anicteric: Outpatients with elevated levels of ALT, AST, or bilirubin and with nausea, vomiting, scleral icterus, or dark urine.

^bIndeterminate: Outpatients were not re-examined; prodromal: hospitalized with jaundice 5 to 30 days (median, 13 days) later; ≤ 7 days and 8 to 15 days: after onset of jaundice or dark urine in inpatients.

^cIEM results: No. with detectable HEV/No. assayed.

^d $P = 0.02$, Fisher exact test, two-tailed.

^e $P = 0.01$.

^f $P = 0.4$.

^g $P = 0.07$.

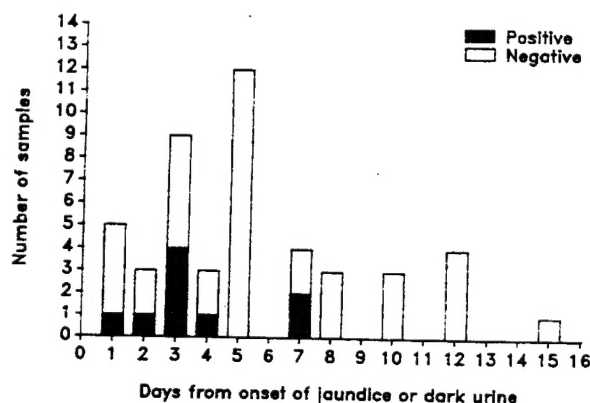


Fig. 5. HEV particles in fecal suspensions and relationship to onset of symptoms of hepatitis. Samples from the Sargodha outbreak were tested for HEV particles by IEM. Each square indicates a single sample (totaled along the ordinate) collected on a day relative to the onset of jaundice or, for 5 patients that did not develop jaundice, dark urine (abscissa). The IEM result for one of these latter 5 patients was plotted on day 5, from the onset of scleral icterus, because the onset of dark urine was not recorded. Positive ■, HEV detected by IEM; Negative □, virus detected.

llected during this period (Fig. 5, Table II). The specimen with the highest concentration of HEV particles was collected 4 days after the onset of dark urine from patient Sar-55. HEV was not detected in the 11 feces collected during the second week of jaundice.

The differences between detectable HEV excretion from patients with acute icteric hepatitis (9/47 positive) and that from all outpatients (1/38), or from those presumed to have remained anicteric (0/26), were not likely to have resulted from random variation (Table II; $P \leq 0.05$ in two-tailed Fisher exact tests). Although HEV particles were detected more often in specimens collected during the first week of the acute phase (9/36 positive) than during the second week (0/11) or prodromal phase (1/12), these differences could have resulted from chance ($P > 0.05$). There was no correlation between

the presence of HEV particles in feces and the level of ALT or bilirubin in serum.

In addition to these 85 patients, 10 individuals had scleral icterus or dark urine but normal levels of ALT, AST, and bilirubin. Their feces were negative for HEV and these results were excluded from analysis in Table II because there was not biochemical evidence of hepatitis.

DISCUSSION

By performing IEM on sera and feces from an outbreak of ET-NANB hepatitis in Sargodha, Pakistan, we demonstrated that patients had been infected with HEV and were excreting the virus. The unusual finding of a seroconversion between the acute and convalescent phases in patient Sar-5 was evidence of infection with HEV during the outbreak. In addition, acute-phase specimens from patient Sar-12 included serum that had anti-HEV with an IgM-like appearance and feces that contained HEV, so it is likely that this patient was also acutely infected with HEV. HEV was detected in one of 12 feces collected during prodromal symptoms but was predominantly excreted during the first 7 days after the onset of icteric hepatitis. Thus, these serologic responses to HEV and the pattern of virus shedding were strong evidence that HEV was etiologically associated with the Sargodha outbreak.

HEV particles in Sargodha feces were serologically identified by using pre-inoculation and acute-phase plasma from an HEV-infected chimpanzee. These particles were morphologically similar to HEV from other sources and distinctly larger (29×31 nm) than hepatitis A virus particles (27×29 nm) photographed on the same day [Ticehurst, 1991]. The 30 nm average of short and long diameters for HEV from Pakistan and Mexico measured by us was larger than our measurement of 29 nm for HEV from Nepal but smaller than that of 32 nm reported for HEV from Mexico, Somalia, Myanmar (formerly Burma), and the USSR by Bradley et al. [1988a]. However, these differences are probably not significant because the indistinct outline of antibody-coated viruses makes precise measurement difficult.

Our conclusion that the virus particles we observed in Sargodha feces were HEV is supported by additional data. When the Sar-55 fecal suspension (Table I, Fig. 4) was inoculated into cynomolgus monkeys, the animals developed hepatitis and anti-HEV. In addition, they shed HEV particles in their bile (R. Purcell et al., unpublished data). The nucleotide sequence of this virus, tentatively designated HEV strain Pakistan '87 or HEV Sar-55 [Ticehurst, 1991], is similar to that of HEV Myanmar '82 [Bradley et al., 1987; Tam et al., 1991; Tsarev, et al., 1992].

Concentrations of HEV in Sargodha fecal suspensions appeared to be very low. We detected <1 to 5 HEV particles per EM grid square, which corresponds to approximately 10^6 HEV particles per gram of feces [Thornhill et al., 1975; Ticehurst et al., 1987]. Although higher concentrations of virus were detected in feces from an HEV-infected volunteer [Balayan et al.,

1983], our results are consistent with those from earlier studies of acute-phase feces from patients in epidemics of hepatitis E [Arankalle et al., 1988; Bradley et al., 1988a,b; Purcell and Ticehurst, 1988].

There are few data for comparison with the pattern of HEV shedding during the Sargodha outbreak, but others have had similar findings. Balayan et al. [1983] observed HEV in serial fecal specimens collected from a human volunteer prior to and during the early phase of nausea, vomiting, fever, dark urine, and elevated levels of ALT, but detectable excretion of virus stopped before the onset of jaundice. Cao et al. [1989, 1991] assayed 60 specimens from 6 patients in Xinjiang Uighur Autonomous Region, PRC, and detected 27- to 32-nm virus-like particles that were predominantly excreted between 4 days prior to and 6 days after the onset of ET-NANB hepatitis. However, it is not known if these particles are serologically related to other characterized strains of HEV and the pattern of their excretion was not related to jaundice. In another study, like ours, of single specimens from different patients, only one of 28 acute-phase feces from an outbreak in India contained detectable HEV [Arankalle et al., 1988].

The pattern of HEV shedding that we observed was different from those of some other enteric viruses. Hepatitis A virus, a picornavirus, is primarily excreted prior to illness and in much higher concentrations than we observed for HEV [Dienstag et al., 1975; Coulepis et al., 1980; Ticehurst et al., 1987]. Norwalk virus particles were present in relatively high concentrations (occasionally ≥ 100 particles per grid square by IEM) in feces from volunteers with gastroenteritis, but virus particles were not detected prior to illness and were detected in only 2 of 11 specimens after 72 hr of gastroenteritis [Thornhill et al., 1975]. In a study of calicivirus infections in children, virus particles were not detected by direct EM of 7 feces collected 2 to 10 days before gastroenteritis but were detected in 25 of 33 feces obtained within 9 days of onset of illness [Chiba et al., 1980].

Possible explanations for the infrequent detection of HEV are related to the epidemiology of hepatitis E and technical limitations. First, it is not understood why hepatitis E occurs in populations that are infected with many enteric viruses during early childhood. Excretion of low concentrations of HEV during a relatively short period of the infection may restrict the spread of the virus. Second, IEM is a relatively insensitive assay, probably detecting HEV only when there are $>10^6$ particles per IEM preparation [Thornhill et al., 1975; Ticehurst et al., 1987]. In addition, it has been suggested that HEV is labile [Bradley et al., 1988b].

Characterization of the virus and seroepidemiologic studies of HEV infections would be much more readily performed with faster or more sensitive assays. A solid-phase IEM method appears to be more rapid and sensitive than standard IEM [Humphrey et al., 1990]. A fluorescent antibody-blocking assay for anti-HEV [Krawczynski and Bradley, 1989] is also more rapid than IEM, but immunofluorescence microscopy cannot be used for detecting extracellular HEV antigen. Sev-

eral prototype immunoassays [Balayan et al., 1983; Bradley et al., 1987; Belabbes et al., 1988; Favorov et al., 1989; Gupta et al., 1988; Liu et al., 1991; Pillot et al., 1987] have not yet been reproduced in other laboratories. It may be difficult to develop rapid immunoassays because few sera have been identified with high titers of anti-HEV IgG, a component of many solid-phase immunoassays, and because feces rarely contain sufficient HEV to be used as an antigenic reagent in such assays. However, bile from experimentally infected cynomolgus monkeys (Figs. 2, 3) [Bradley, 1990a,b; Bradley et al., 1990; Uchida et al., 1990] and cloned cDNA [Reyes et al., 1990; Yarbough et al., 1991] may be better sources of antigens. Cloned cDNA is also a source of reagents for hybridization methods, including the polymerase chain reaction.

Although IEM is cumbersome and insensitive, its visual specificity assures confidence in the results [Kapikian et al., 1976]. Thus, IEM continues to be the most reliable assay for both HEV and anti-HEV. Analysis of ET-NANB hepatitis has been facilitated by the identification of a single antigenic group of virus, as defined by IEM, and by the demonstration of anti-HEV in every natural and experimental case tested to date [Arankalle et al., 1988; Asher et al., 1990; Balayan et al., 1983, 1990; Bradley et al., 1987, 1988a,b; Krawczynski and Bradley, 1989; Purcell and Ticehurst, 1988; Sjogren et al., 1987; Sreenivasan et al., 1984; Ticehurst, 1991] (B. Innis et al., J. Ticehurst et al., unpublished data). However, the possibility of additional serotypes of HEV or other agents [Doroshenko et al., 1989; Kuo et al., 1989] should be considered in evaluating each case of epidemic or sporadic NANB hepatitis.

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